UNIVERSITY OF ALBERTA

# FORMULATION OF TARGETED LIPOSOMES FOR THE ORAL DELIVERY OF POORLY-ABSORBED DRUGS

BY

KEITH E. ANDERSON ©

A THESIS

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"Mediocre spirits demand of science the kind of certainty which it cannot give, a sort of religious satisfaction. Only the real, rare, scientific minds can endure doubt, which is attached to all of our knowledge"

-Sigmund Freud

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## Abstract

The application of liposomes in the oral delivery of poorly-absorbed, water-soluble drugs has had limited success to date. Issues of stability and specific uptake by the gastro-intestinal tract require attention. Hence, a multi-faceted study was initiated to address in vitro requirements of formulation design and in vivo performance in a rat model.

A strategy of liposome surface coating by a polymer-modified nutritional factor was pursued. Some physicochemical aspects of phospholipid monolayer behavior were examined at the chlorobenzene/water interface and quantitative changes in monolayer characteristics led to the development of a polymer impact ratio, from which the degree of change in monolayer integrity could be predicted.

The targeting strategy employed hydrophobically-derivatized folic acid (folic acid-poly(ethylene oxide) (PEO)-cholesterol) adsorbed at liposome surfaces, designed to improve the intestinal uptake of liposomes through folic acid-receptor-mediated endocytosis. The derivatized folic acid retained specificity for binding receptors, improved the transport of PEO 3350 MW across Caco-2 cells 8-fold compared to underivitized PEO-bis(amine), and, when employed as a liposome surface-modifier, improved the transport of liposome-entrapped Texas Red dextran 3000 MW across Caco-2 cells 5-fold.

DSPC:CH:DCP:FA-PEO-CH (3:1:0.25:0.05 mole ratio (m.r.)) was identified as an optimized liposome system for the oral delivery of a model glycopeptide vancomycin. Liposomes were prepared as dehydration-rehydration vesicles at an average size of 200 nm resulting in an encapsulation efficiency for vancomycin of

25 percent, of which 65 percent remained entrapped after exposure to simulated intestinal fluids containing 10 mM bile salts solution for 2 h at 37°C. In contrast to Texas Red dextran, vancomycin was not transported across Caco-2 cells to any appreciable extent when loaded in targeted liposomes, although 14 percent of the dose was indirectly determined to be delivered intracellularly.

In vivo, a 3.9- and 12.5-fold increase in bioavailability of vancomycin was found using non-targeted liposomes (6.7 percent bioavailable) and targeted liposomes (21.8 percent bioavailable), respectively when compared to a vancomycin solution (1.74 percent bioavailable). The potential for development of an oral peptide delivery system based on folic acid-targeted liposomes was realized.

This manuscript is dedicated to my wife Lise and our respective families, with whose constant support was the resource necessary for completion of this work.

## Preface

Recent developments in liposome technology have created a rekindled interest in the utility of these systems for the oral delivery of poorly-absorbed agents. It is the intent of the following investigations to implement sound formulation and drug delivery practices to develop a functional liposome dosage form to improve the oral bioavailability of a model peptide. In particular, strategies aimed at gastrointestinal stability and improvement of oral absorption are described.

**Acknowledgements**

The work described in this thesis is the result of many successful collaborative efforts which are responsible, in large part, for the completion of this multi-faceted project. In no particular order, I personally want to thank the following groups of people.

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## List of Abbreviations / Nomenclature

**Liposome Contents:**

| | |
|---|---|
| CH | cholesterol |
| DCP | dicetylphosphate |
| DLPC | dilaurylphosphatidylcholine |
| DMPG | dimyristoylphosphatidylglycerol |
| DPPC | dipalmitoylphosphatidylcholine |
| DPPE | dipalmitoylphosphatidylethanolamine |
| DSPC | distearoylphosphatidylcholine |
| EPC | egg phosphatidylcholine |
| HEPC | hexadecylphosphocholine |
| HSPC | hydrogenated soy phosphatidylcholine |
| PA | phosphatidic acid |
| PC | phosphatidylcholine |
| PE | phosphatidylethanolamine |
| PI | phosphatidylinositol |
| PS | phosphatidylserine |
| SA | stearylamine |
| SG | soy-bean derived glucosides |
| SPC | soy phosphatidylcholine |
| SS | Soy-bean derived sterols |

**Manufacturing Methods:**

| | |
|---|---|
| DRVs | dehydration-rehydration vesicles |
| FATMLVs | freeze and thaw multilamellar vesicles |
| LUVs | large unilamellar vesicles |
| MLVs | multilamellar vesicles |
| REVs | reverse evaporation vesicles |
| SUVs | small unilamellar vesicles |

**Drugs and Chemicals:**

| | |
|---|---|
| AL | asparagus pea lectin |
| Ara-C | cytarabine arabinoside |
| AZT | azidothymidine (zidovudine) |
| AZT-CDS | azidothymidine chemical delivery system |
| $BH_4$ | tetrahydrobiopterin |
| BSA | bovine serum albumin |
| CHD | cholesterol-dervitized dextran |
| CHM | cholesterol-derivitized mannan |
| CHP | cholesterol-derivitized pullulan |
| CM-chitin | carboxymethylchitin |
| conA | concavalin A |
| CT | cholera toxin |
| CTAB | cetyltrimethylammonium bromide |
| CTB | cholera toxin B-subunit |
| DMF | dimethylformamide |
| DMSO | dimethylsulfoxide |
| DOX | doxorubicin |
| DT | diptheria toxin |
| EPO | erythropoietin |
| FA | folic acid |
| FITC | fluorescein isothiocyanate |
| HRP | horse radish peroxidase |
| ML | mycoplasma gallisepticum lectin |
| NaC | sodium cholate |
| NaCDC | sodium chenodeoxycholate |
| NaDC | sodium deoxycholate |
| NAcGal | n-acetylgalactosamine |

| | |
|---|---|
| NSAIDs | non-steroidal anti-inflammatory drugs |
| OPP | o-palmitoyl pullulan |
| OVA | ovalbumin |
| PAA | poly(acrylic acid) |
| PEG | poly(ethylene glycol) |
| PEGylated | PEG-coated |
| PEO | poly(ethylene oxide) |
| PET | poly(ethylene terphalate) |
| PVA | poly(vinyl alcohol) |
| PVP | poly(vinyl pyrrolidone) |
| $\sigma 1$ | reovirus capsid protein |
| SOD | superoxide dismutase |
| TR-dex | Texas Red Dextran 3000 MW |
| TT | tetanus toxoid |
| UEA-1 | ulex europaeus I |
| WGA | wheat germ agglutinin |
| VCM | vancomycin |
| vit $B_{12}$ | vitamin $B_{12}$ (cyanocobalamin) |

**Miscellaneous:**

| | |
|---|---|
| ADSA | axisymmetric drop shape analysis |
| CMIS | common mucosal immune system |
| FAE | follicle-associated epithelium |
| GALT | gut-associated lymphoid tissue |
| GI tract | gastrointestinal tract |
| HBS | Hepes buffered saline |
| IR | infrared |
| i.v. | intravenous |
| M-cells | microfold cells |

| | |
|---|---|
| MPS | mononuclear phagocyte system |
| NMR | nuclear magnetic resonance |
| PBS | phosphate buffered saline |
| $PGE_2$ | prostaglandin $E_2$ |
| PHA | phytohemagglutinin |
| PPs | Peyer's patches |
| $R_e^c$ | critical solubilizing ratio |
| RIP | ribosome inactivating protein |
| RME | receptor mediated endocytosis |
| s.c. | subcutaneous |
| SIF | simulated intestinal fluid |
| SIF+ | simulated intestinal fluid with 10mM NaC |
| SIF+BSC | simulated intestinal fluid with 10mM bile salt cocktail |
| SGF | simulated gastric fluid |
| SPB | Sørenson's phosphate buffer |
| TER | transepithelial electrical resistance |
| TL | tomato lectin |
| $T_m$ | phase transition temperature |
| $TXA_2$ | thromboxane $A_2$ |
| USP | United States Pharmacopoeia |
| ZO-1 | zonula occuldens-1 tight junction protein |

**Pharmacokinetic Parameters:**

| | |
|---|---|
| AUC | area under the curve |
| Cl | clearance |
| Cl/F | oral clearance |
| $C_{max}$ | maximum concentration |
| F | bioavailability |
| LPR | lymph to plasma ratio |

| | |
|---|---|
| $P_{app}$ | apparent permeability |
| PK | pharmacokinetic(s) |
| $t_{1/2}$ | half-life |
| $t_{max}$ | time to maximum concentration |
| $V_d$ | volume of distribution |

**Physico-chemical Characterization:**

| | |
|---|---|
| EE | encapsulation efficiency |
| $P^*$ | polymer-impact ratio |
| $\pi$ | surface pressure |
| $\gamma$ | interfacial tension |

## *Chapter 1*

# INTRODUCTION

---

A version of this chapter has been accepted for publication in Critical Reviews of Therapeutic Drug Carrier Systems.

## 1.1 Introduction

The oral delivery of potent, specific biologicals, particularly proteins and peptides, continues to be a pursuit for the 'Holy Grail' of drug delivery technologies. A variety of proposed approaches have emerged over the past decade to improve the oral bioavailability of this class of compounds, ranging from chemical modification to protein-loaded colloidal delivery systems. Oral liposome delivery systems have been advocated with limited success, particularly with regard to reproducible dose-to-dose bioavailability, a pre-requisite for the delivery of potent therapeutic agents. Recent liposome technologies have focused on strategies to improve the stability of these vesicular systems in the gastro-intestinal (GI) tract and the bioadhesive properties of liposome surfaces for the walls of the GI tract. This thesis describes the formulation of a surface-modified, targeted oral liposome system designed to exploit folic acid (FA)-mediated transcellular routing for systemic delivery of a model glycopeptide, vancomycin (VCM). The following background sets the stage for the development of such systems, and a version of which has been accepted as a review in Critical Reviews in Drug Carrier Therapeutic Systems (1).

## 1.2 Oral Liposome Delivery

Liposomes have been advocated in many applications, particularly in the medical and cosmetic fields. For the most part, liposomes serve as carriers of therapeutic and cosmetic agents but by virtue of their composition, being predominantly phospholipids, their effectiveness has also been demonstrated as penetration enhancers as well as skin modifiers. To date, liposomal dosage forms in clinical use have been limited to topicals and parenterals, although, as early as 1976 (2;3), the potential of liposomes to increase the oral absorption of entrapped agents had been explored with some apparent success. However, a number of subsequent reports involving entrapped insulin led to confusion and outright contradiction of liposomes as a useful oral dosage form (4-9). Nevertheless, the mood has been changing and new developments and knowledge of the oral absorption processes have provided clues to improved approaches in formulation of oral liposomes (and other microparticulates) that could successfully deliver therapeutic amounts of entrapped agents.

Early attempts at oral liposome therapy focused mainly on insulin due to its clinical relevance and ease of measuring therapeutic endpoints in diabetic animals, although blood glucose changes in normal animals were ambiguous at best. A concern during this period was the relatively low entrapment efficiency of insulin, between 9-11percent. Nevertheless, blood glucose levels decreased after intragastric administration of liposomal insulin, positively correlating with the length and saturation of the fatty-acid chains of the phosphatidylcholines (PC) (2;6). Also, when (+)-tubocurarine was orally-administered in liposomes rats became sick or died in 2 h, with evidence of (+)-tubocurarine in the blood associated with liposomes (6). Heparin, Factor VIII and IX also appeared to be encouraging candidates for oral liposome delivery (10;11). In spite of these early successes by some, several other researchers have not found oral liposomes as promising (8;9;12). It has been argued that even the most stable liposome formulations, e.g. distearoylphosphatidylcholine:cholesterol (DSPC:CH, 7:4 m.r.) are susceptible to rupture by bile salts with subsequent leakage of entrapped agent (13), and liposomes composed of nondigestible, stable phospholipids were no more effective (12). In rat gut sac experiments, both entrapped hydrophilic marker and liposomes were found to transport across the tissue and reach serosal fluid (14-16). A 26-fold increase in transport was found and the process was dependent upon the concentration of liposomes. In addition to his own studies, Woodley had previously reviewed other outcomes of oral liposome drug delivery (17).

Liposomes may be classified as microparticulates (18) normally within the colloidal size range. Thus, characterization of liposomes with respect to formulation, absorption, or elimination falls within the general scope of behavior regarding protein or polymeric micro- and nano-particulates, which also have been afforded considerable attention as oral drug delivery systems. Table 1-1 summarizes some of the features of particulate systems which need to be considered in the developmental process. In addition, O'Hagen (19) has emphasized that microparticulate uptake observed in animals is influenced by the fed state and age of animal, the species under investigation, and the method used to quantify uptake. Not suprisingly, many reports of oral liposome delivery have not addressed all of these factors, which has led to variability in results without the understanding needed to re-address the discrepancies. A more methodical process of development is now underway that attempts to gain an understanding of physical, chemical, and biological mechanisms involved between, i) encapsulated agent and liposome structure, ii) liposome composition and integrity in various gastrointestinal media, iii) liposome surface architecture, targeting moieties and target site, iv) liposomes and the epithelial lining at absorption sites and consideration of the fate of drug (or drug-liposome complex) once transport across the gut wall has occurred. In this regard, there are clearly different outcomes depending on which tissue or cell-type is involved, for example stomach versus intestine and enterocytes versus Microfold (M)-cells within the jejunal and ileal segments of the small intestine.

## 1.3 Systemic Delivery via Oral Liposomes

### *1.3.1 Mechanistic Studies*

Prior reviews (17;20;21) of oral liposome administration have been helpful in highlighting several issues of importance, including the noted complexity of experimental design, variability in response, multiplicity of formulation variables, and stability problems in the GI tract. Gradually, an understanding of liposome uptake at the cellular level is beginning to emerge. Woodley et al. (15;16) adapted an everted gut sac system to elucidate some mechanistic aspects of the intestinal transport of liposome-associated poly(vinyl pyrrolidone) (PVP), horseradish peroxidase (HRP), and insulin. Appearance of the liposomes and contents on the serosal side of the intestinal sacs was clearly evident, although only as a small fraction of the lumenal concentration. Transport was saturable and sensitive to metabolic inhibitors, suggesting adsorptive endocytosis as a primary means of uptake. This led to the postulate that adsorptive endocytosis could be augmented by attaching ligands to the liposomes having an affinity to the intestinal epithelium. It was also determined that smaller liposomes (<0.5 μm) were transported into cells 4-5 times faster than larger liposomes, however this was offset by lower encapsulation efficiency and less stability within the GI tract. Patel et al. (22) have disputed the findings of the gut sac experiments, arguing that absorption via the vascularly perfused isolated intestinal loop was a more physiologically relevant indicator of liposome uptake. Even though liposomes were found to be internalized by the gut wall, they were degraded intracellularly, preventing the appearance of liposomes in the venous effluent. Schwinke and Weiner (23) observed optimal uptake rates for the more stable DSPC and DSPC:CH (2:1 m.r.) liposomes ($t_{1/2}$= 19 & 21min, respectively) using an in situ jejunal loop model, however they failed to find evidence of significant systemic tissue levels of $^{14}$C-lipid or entrapped markers ($^{14}$C-PEG 4000, $^{14}$C-glucose). The uptake process followed linear kinetics and was dependent on the lipid phase transition temperature. Uptake measurements, accounted for by loss of liposomes from the isolated intestine, represented the coincidental processes of epithelial adsorption and transport. Dual-labelled liposomes ($^{3}$H-CH, $^{14}$C-PC) ruled out lipid exchange with intestinal cell membranes, thus it was concluded that the uptake processes involved intact liposomes. These results suggested that liposomes may adhere to the jejunal mucosa but do not improve absorption of poorly absorbed compounds. Later work (24) revealed that using the hydrophilic markers PEG-4000, inulin, and insulin can result in coating of liposome surfaces, suppressing uptake and preventing any interaction with the intestinal loop. The implication of this for oral liposome delivery is that the architecture of liposome surfaces, determined by formulation procedures or surface films, plays an important role in determining their attachment to epithelial surfaces. Subsequent work by Chiang and Weiner (25;26) have left the impression that liposomes have essentially little potential at improving the absorption of entrapped agent after oral administration. Thus, findings from mechanistic studies do not at this time fully account for the variability in observations of oral liposome therapy (17;27), and suggest that certain unaccounted-for-mechanisms have yet to be identified. For instance, the lymphatic route of absorption may be more significant than previously thought.

### *1.3.2 Pharmacokinetic Studies*

Absorption of orally-administered liposome-entrapped agents in the animal model is generally evaluated by appearance of compound in the systemic circulation or by its pharmacological action, for example the lowering of blood glucose levels, blood clotting times, etc. The bioavailability is dependent upon a series of inter-related factors, including liposome loading, dose, amount retained at absorption site(s), anatomy and physiology of the absorption site(s), and the pharmacokinetics (PK) involved. A misleading assumption prevalent in some PK studies is that the clearances of free and liposome-entrapped drug from plasma are equivalent, even though the elimination, including renal clearance, of drug-associated colloids and free drug are known to be radically different (28), largely due to macrophage uptake of colloidal particles. Also, after absorption into the portal circulation, the fate of liposomes is tempered by liver extraction, which usually results in total destruction of liposomes, or their biliary excretion and elimination. Hence, if cumulative urinary excretion is the only data collected, prior events related to liposome absorption and the resultant higher plasma levels of entrapped drug may be obscure. In order to meet the experimental objective of determining liposomal transport across the GI tract, measuring portal vein blood levels of liposomes or drug should give a more accurate result. Alternatively, a liposome-entrapped, water-soluble compound which does not undergo significant first-pass metabolism or lumenal degradation, e.g. VCM, could be utilized to demonstrate the potential of liposomes for improving oral absorption, thereby eliminating complicated cannulation procedures. A compilation of relevant reports of oral liposome delivery appears in Table 1-2.

Evidence of the role of liposome composition on bioavailability from the GI tract is growing. A report involving insulin absorption after its oral administration in 7:7:1 m.r. dipalmitoylphosphatidylcholine:CH:dicetylphosphate (DPPC:CH:DCP) liposomes indicated greater insulin stability and appearance in the portal blood than from 7:2:1 m.r. liposomes at 3 h post-administration as determined with radioactive insulin (29). Sixty and 15 percent of radioactive insulin in plasma was associated with CH-rich and CH-poor liposomes, respectively. An extended duration of insulin absorption was observed for CH-rich liposomes with no evidence of liposomes in cardiac blood. A two-fold increase in the absorption of a larger protein, egg ovalbumin (OVA, ca. 45,000 MW), in negatively-charged egg phosphatidylcholine (EPC) liposomes has been reported (30). Rifampicin administered orally to rats in liposomes produced significant changes in post-absorptive systemic distribution when compared to free rifampicin (31). This was explained as possibly being due to some of the dose being transported by the lymphatics resulting in a significant prolongation of time to maximum concentration ($T_{max}$). Several liposome formulations of oil-soluble vitamins for oral delivery have appeared in the literature. In comparison to conventional formulations, liposomes increased blood levels and liver uptake of $\alpha$-tocopherol (32). Negatively-charged gel-state lipids performed the best. Liver uptake of vitamin D was also improved, but uptake of vitamin A was not. Systematic in vitro stability studies of fat-soluble vitamin liposome formulations (33) revealed that multi-vitamin (A, $D_3$, E) incorporation stabilized the vesicle membrane at an optimal encapsulation ratio of 0.5:1 (vitamin:lipid m.r.), and stability against bile salt-induced rupture was positively correlated with the phospholipid phase-transition temperature ($T_m$). A brief report alludes to the enhancement of oral bioavailability and pharmacodynamic (PD) effect of novel water-insoluble drugs in 400 nm EPC liposomes, but no explanation of the results was forthcoming (34). The poorly absorbed vitamin, tetrahydrobiopterin ($BH_4$), given orally in DSPC liposomes exhibited a 50 percent increase in plasma levels (35) but with no apparent differences in the elimination kinetics of free and liposomal $BH_4$. On the other hand, oral griseofulvin delivery was improved by liposome encapsulation conferring a 2.6-, 3.3-, and 2.1-fold increase in maximum concentration ($C_{max}$), area under the curve (AUC), and half-life ($t_{1/2}$), respectively (36). The differences in the elimination kinetics of free- and liposomal-griseofulvin suggest that liposomes were associated with griseofulvin in blood. AZT-CDS, a novel brain-selective chemical delivery system (CDS) for zidovudine, when formulated in 100 nm EPC liposomes and delivered intrajejunally increased blood and brain levels of AZT by 6- and 35-fold, respectively, compared to an equal dose solubilized in dimethylsulfoxide (DMSO). AZT-CDS is sensitive to the acidic pH of the stomach and normally undergoes first-pass metabolism. Hence, its liposome association appears to be responsible for the improvement in bioavailability resulting from gastric protection and reduced first-pass metabolism (37). Furthermore, improved distribution of AZT to treatment sites in the brain after oral liposome delivery suggested that AZT in the systemic circulation was associated with an enhancer, possibly liposomes. The potential of oral liposome formulations of novel protease inhibitors for the treatment of HIV-1 infected individuals has been recently expressed (38). In addition to increasing oral bioavailability, liposomes also target these agents to macrophages, which are major reservoirs of HIV-1.

Recombinant human erythropoietin (EPO), a glycoprotein (ca. 30,000 MW), was encapsulated in reverse evaporation vesicles (REVs) and administered orally to rats (39). EPO-liposomes 100 nm in size were absorbed to a greater extent than 200 nm liposomes. In addition to size dependency, 100 nm EPO-liposome compositions of DPPC and soybean-derived sterols (SS), CH, or soybean-derived glucosides (SG) followed uptake in the order DPPC:SS (7:2 m.r.) > DPPC:CH (7:2 m.r.) > DPPC:CH (7:4 m.r.) = DPPC:SG (7:2 m.r.), indicating that more rigid vesicles allowed greater absorption of EPO. In-depth studies revealed that inclusion of SS was more effective than CH in stabilizing the liposomes in blood (40), perhaps because SS yielded a higher $T_m$ and, therefore, lower fluidity of the liposomes (41). On this basis, the reason for a poorer performance of DPPC:CH (7:4 m.r.) liposomes was not apparent. The EPO-liposomes were absorbed across the intestinal mucosa in pharmacologically active form, but exhibited long lag-times and sustained activity from which it was concluded that the liposomes had become trapped at the absorption site. Absorption via a lymphatic pathway, such as by chylomicrons or through Peyers patches (PPs) is a possible explanation of this behavior. Absolute bioavailability of EPO:DPPC:SS liposomes ranged from 1-31 percent (39), obviously a considerable variability. Use of this optimum liposome composition in oral insulin delivery also improved its availability to the blood and the lag-time for glucose reduction was longer, this time in the order DPPC:SS (7:4 m.r.) > DPPC:SS (7:2 m.r.) > DPPC:CH (7:4 m.r.) >> DPPC:CH (7:2 m.r.) liposomes. The pharmacological availability of insulin from the DPPC:SS (7:4 m.r.) liposomes reached 31.6 percent and lower blood glucose levels were maintained for at least 21 h (42). An interesting aspect of this work was the discovery that the body weight of rats fed empty SS- and SG-containing liposomes increased, the more rigid bilayer of the SS composition making the greatest impact. When SS and SG lipids were simply administered in aqueous suspension no weight gain was observed (43). Perhaps it could be argued that this was fortuitous, however a PK analysis of hexadecylphosphocholine (HEPC, Miltex®), a novel chemotherapeutic agent for treatment of mammary carcinomas and skin metastasis, might serve to dispel any doubt (44;45). Free HEPC is completely absorbed by the intestine, likely by passive diffusion via chylomicrons and lymphatic capillaries, reaching the systemic circulation via the thoracic duct. Formulation of 70 nm HEPC small unilamellar vesicles (SUVs) containing CH and dimyristoylphosphatidylglycerol (DMPG) resulted in a sustained release profile of HEPC in plasma. The extent of HEPC in plasma increased 40 percent, $C_{max}$ was reduced by 36 percent, $t_{1/2}$ increased 2.5 times, total body clearance was reduced by half, and the volume of distribution ($V_d$) doubled. There was no reduction in body weight, no incidence of gastrointestinal lesions, or ruffled skin, which are toxic symptoms associated with free HEPC. In comparison, i.v. administration of free and liposomal HEPC yielded reversed kinetic profiles, liposomal HEPC now being cleared rapidly from the blood probably by the reticulo-endothelial system (RES).

Encapsulation of superoxide dismutase (SOD) by DSPC:CH: stearylamine (SA) liposomes resulted in 20 percent relative oral bioavailability compared to the subcutaneous route (46). Incorporation of ceramides into the liposomes further enhanced bioavailability to ~60 percent. Oral delivery resulted in sustained levels of systemic SOD peaking at 5 h, suggesting that liposomes provided a depot of drug. Protection of SOD from enzymatic degradation (46;47) was suggested to be the primary reason for absorption enhancement, although it remains to be determined whether such liposomes adsorb to cell surfaces, fuse with cell membranes, undergo endocytosis or a combination of these events. Extension of this SOD delivery system into in vivo inflammatory animal models resulted in significant anti-inflammatory action, and was accompanied by reductions in cellular $PGE_2$- and $TXA_2$-synthase activities (48). Since circulating free SOD is not correlated with anti-inflammatory activity, the authors suggested that ceramide incorporation in SOD liposomes facilitated drug activity by modulating cellular transduction mechanisms, thereby making the assumption that circulating SOD was associated with liposomes after oral delivery. Considering the extent of uptake, oral liposomes have produced relatively high bioavailabilities, lending argument for systemic therapy, previously thought untenable by oral liposome administration.

### *1.3.3 Pharmacodynamic (PD) Studies*

Testing the effectiveness of an oral liposome formulation is often most meaningful by monitoring a PD event, such as blood glucose lowering by insulin. Choudhari et al. (49) found that increasing the lecithin:insulin ratio yielded enhanced delivery up to a maximum of 100 to 200 mg lecithin in rabbits. Incorporation of CH reduced the efficacy of liposomal insulin, whereas addition of 1 percent Tween-80 enhanced delivery. The activity of the administered liposomal insulin was sustained for a longer period of time in diabetic compared to non-diabetic rabbits. Whether liposomes were prepared by the classical hydration or solvent-spherule-evaporation method made little difference, each conferring about 1 percent of the activity of a s.c. dose of insulin. When EPC and DPPC liposomes containing either phosphatidylinositol (PI) or dipalmitoylphosphatidylethanolamine (DPPE, 1:1 m.r.) were loaded with insulin, hyperinsulinemia was recorded, however blood glucose reduction due to biologically-active insulin only occurred with the DPPC liposomes (50). Using chitosan-coated insulin liposomes blood glucose levels were reduced 5-10 percent of that obtained from the s.c. route, a significant result and approximately a 5-fold reduction compared to uncoated insulin liposomes (51). The prolonged reduction (>12 h) of blood-glucose was attributed to accumulation of liposomes at absorption sites due to bioadhesiveness of the chitosan-coating (51;52). Poly(ethylene glycol) (PEG)-coated liposomes also enhanced oral delivery of insulin maintaining reduced blood glucose levels for up to 8 h. The explanation of this result was improved stability of the liposomes in the presence of bile salts with little insulin leakage and increased interactions with the intestinal epithelium presumably due to the extended PEG chains (53), contrary to previous observations (24). However, mechanistic studies are required to confirm this. The uptake of a model peptide, calcitonin, in rats after oral administration in EPC liposomes was augmented and its persistence in blood was increased although not to a large degree (54). On the other hand, liposome-associated indium ($^{111}In^{3+}$) given in a similar fashion did not appear in blood, possibly because the liposomes were degraded intracellularly preventing transcytosis of $^{111}In^{3+}$, a finding corroborating a previous report of intracellular liposome degradation (22). Mass-balance recovery indicated that the residence time of $^{111}In^{3+}$ in faeces was prolonged for an extra day after oral liposome feeding, coinciding with the average turn-over times of intestinal epithelial cells (2-3 d). Poor stability of the EPC liposomes to bile salts was recognized as a limiting factor for oral drug delivery. Generally, unsaturated liposomes are less stable than liposomes of saturated phospholipids in the GI tract, thereby the former delivering smaller amounts of drug to absorption sites. However, this did not appear to be the case in earlier work where the delivery of calcitonin from dimyristoylphosphatidylcholine (DMPC) or DPPC liposomes was found to be less than from EPC liposomes (55). In comparison, the inclusion of SA in the liposome bilayer appeared to be a determining factor for both calcitonin and parathyroid hormone delivery. SA was also a determinant in optimized liposome formulations of streptokinase in a collection of works describing the stability, preparation technique, characterization and enhanced in vivo activity (56-58). Factor VIII orally-administered in multi-lamellar vesicles (MLVs), REVs, or dehydration-rehydration vesicles (DRVs) to haemophilic dogs (59) yielded no changes in clotting behavior, possibly because of poor encapsulation, the use of low $T_m$ PCs, and the labile nature of Factor VIII to acidic environments. Although DSPC liposomes prepared as DRVs were found to have reasonably high encapsulation of Factor VIII, these were, unfortunately, not tested in vivo. Oral liposome delivery of Factor VIII has been successful in humans (60;61), and incorporation of PEGylated Factor VIII in liposomes extended its duration in plasma. Sakuragawa et al. (62) has also reported administering liposomal PEGylated Factor VIII and IX together with the protease inhibitor, aprotinin, to patients and obtained evidence of systemic activity.

Chickens have been used as an oral absorption model for testing liposomal uricase in the treatment of gout (63). Results showed a 50 percent reduction of blood uric acid accompanied by a rise in plasma uricolytic activity. Liposome encapsulation was also responsible for improvements in the narcotic response to the opiate antagonists, morphine and fentanyl, after oral administration to rats (64). Albendazole, an anthelminthic used for treatment of human hydatidoses, such as cystic and alveolar hydatidosis, exhibits low bioavailability and erratic uptake from the gut. Pharmacodynamic analysis of parasite burden after infection with *E. multilocularis* and treatment with combination therapy of oral liposomal albendazole and cimetidine provided evidence that improved bioavailability significantly enhanced the therapeutic effect (65), although some of the activity may have been due to distribution of liposomal albendazole to lymphatic tissue.

## 1.4 Pathways and Processes of Gastro-Intestinal Absorption

### *1.4.1 Gastro-intestinal Transport*

Transport across polarized epithelia with tight junctions can be loosely categorized into paracellular and transcellular transport, with molecules in excess of 400 MW unable to be quantitatively transported via the paracellular route. Paracellular transport may be increased through the use of penetration enhancers, chelating agents, osmotic gradients, etc. but even under these conditions diffusion of molecules above 1kD is limited (66;67). Pore radii of ileum and jejunum epithelia have been estimated to be 0.3-0.4 nm (equal to the size of a mannitol molecule, 182 MW) and 0.7-0.9 nm, respectively (68;69). Alternatively, it has been proposed that the occurrence of intercellular spaces and defects in the intestinal mucosa due to sloughing of absorptive cells at the villous tips may allow particles to pass through the intestinal wall by the paracellular route, which again may be enhanced by the presence of non-specific penetration enhancers. Paracellular trafficking is the only reasonable explanation for the rapid time-scale of particulate uptake noted by some investigators (70-73). An extensive histological study using poly(cyanoacrylate) nanospheres (165 nm) loaded with Lipiodol, an iodized-oil, found evidence of transport via the paracellular route enabled by defects in the intestinal mucosa caused by the desquamation of mature villous cells (74). Also, fenestrations appearing in the basal lamina of enterocytes (500-5000 nm) and in endothelial cells (5-100 nm) allowed the nanospheres to enter the systemic circulation.

Hydrophilic macromolecules or association colloids are actively transported via endosomal routing to the basal surface of the epithelial cell layer in order to reach the lymphatic or systemic circulation (68;75-77). Delivery of drug as a result of liposome fusion with cellular membranes has been ruled out as a significant delivery route (23;78). Pinocytosis (fluid-phase endocytosis) is an active process by which cells constantly sample the external environment, however this is also considered to be an inconsequential route for drug delivery (79;80). Receptor-mediated endocytosis (RME) is a means of cell entry which bodes well for efficiency of delivery if site-specificity is achieved, more so than any other endosomal route (81-83). Similarly, adsorptive endocytosis can also result in high efficiency if binding sites exist on the membrane to which the particle has a high affinity (81;82;84;85). Adsorptive endocytosis of molecules which adhere to the apical surface of M-cells, such as ferritin, lectins, HRP, IgG, and the cholera toxin B sub-unit (CTB), appear within minutes on the baso-lateral surface of the M-cell or in the sub-epithelial intercellular space (84;85). Factors governing endosomal routing within the cell determine whether the endosome is merged with a lysosomal compartment, re-routed back to the apical surface, dispersed within the cytoplasm (potocytosis), or transported to the basal surface of the cell (transcytosis) (86). Molecules or particles involved in transcytosis must avoid cytoplasmic and lysosomal enzymatic activity in order to remain functional. Targeted liposomes bearing monoclonal antibodies or protein A were endocytosed according to the target cell type, liposome size, and the surface density of targeting molecules (78). Mechanisms of endosomal routing are variable among different cell-types and routing is dependent on how a cell responds to binding of a targeting molecule and, more importantly, how a cell responds to binding of multiple targeting molecules which, in turn, are bound to a liposome surface. Low et al. (87) have demonstrated plasmalemmal vesicle (caveolae)-mediated endosomal uptake of 120-130 nm liposomes targeted to KB cells, a naso-pharyngeal cell line, and were able to deliver therapeutically significant levels of drug (88). Studies involving an isolated vascularly perfused rabbit ileum revealed that liposome uptake was dependent on phospholipid chain saturation, surface charge, and particle size. Smaller, negatively-charged liposomes consisting of higher $T_m$ lipids were preferentially internalized but, also, those containing the higher $T_m$ lipids were metabolized to a greater extent than those of lower $T_m$ lipids, which limited transcytosis of liposomes to the venous effluent. This is strongly suggestive of a different mode of phospholipid degradation intestinally and intracellularly (22). Based on these precedents, the successful oral delivery of agents by liposomes will require detailed knowledge of enterocyte function, particularly with regards to the endocytotic and transcellular routing of colloids. Also, capacity and control mechanisms of the endocytotic pathway of the specialized cell types of the GI tract will need to be better understood.

RME is described as any event in which the binding of an external substance to the surface of a cell leads to its internalization (83). RME can be a favorable or unfavorable event for a cell, relating to the cellular extraction of beneficial compounds from the external media through receptor expression on the one hand, or to the ability of certain undesirable external substances to gain access to the cytoplasm of the cell through binding to surface ligands on the other, the latter sometimes being regarded as adsorptive endocytosis. Some natural metabolites, nutrients, antibodies, and growth factors which undergo transcytotic routing by RME include low-density lipoprotein (LDL), bile salts, cyanocobalamin (vit $B_{12}$) , pteroylglutamic (folic) acid, epidermal growth factor, nerve growth factor, ferritin, and the polymeric immunoglobulin receptor (pIgR) (68;79;89-91). pIgR is an example of a well-characterized large molecular weight protein that undergoes secretory transcytotic cellular transport across enterocytes (86). Dietary lectins, viruses, bacteria, and toxins are examples of other substances processed by transcytotic RME routing. Utilization of these RME target molecules for oral drug delivery may also provide site-specific targeting to intestinal cell types, transcytosis for systemic therapy, and prolonged GI transit times due to receptor-ligand binding (e.g. lectins) (83). The RME process embodies two major endocytotic mechanisms including clathrin-coated pits and non-coated caveolae. The clathrin-coated pit is the more well-characterized of the two, although it appears that the routing mechanisms of the caveolae-associated pathway may make it a preferable targeting choice for oral drug delivery (79;86;90;92). Caveolae are not only involved in pinocytotic activity, cellular drinking and environmental sampling, but are also involved in many cellular regulatory processes including endocytosis, transcytosis, and potocytosis (79;90;92;93). Potocytosis requires, i) molecular association with caveolae, ii) internalization and recycling of the molecule within 1 h, iii) that uptake is independent of temperature, and iv) that no degradation/metabolism occurs after internalization (90). Examples of molecules that have been shown to undergo endocytotic macromolecular uptake via caveolae include: 5' nucleotidase, albumin, alkaline phosphatase, ferritin, cationized-ferritin, HRP, ricin, conA, CT, LDL, lipoprotein lipase, viruses, GPI proteins, cytoplasmic signaling molecules (src-like kinases, hetero-trimeric G-proteins, and members of the Rap family of small GTPases) and human lymphocyte class I antigens(79;90;91;93-97). It is as yet unclear whether there are subsets of caveolae responsible for different types of intracellular routing. The ability of this vesicular routing mechanism to concentrate molecules, to have selective uptake, to have multifunctional capabilities, and to result in non-lysosomal directed endosomal compartments for transcellular transport make this a highly favorable route for macromolecular and colloidal particle drug delivery (79;90;92;93).

In adult mammals, PPs associated with the follicle-associated epithelium (FAE) appear to be the predominant site for macromolecular and particulate uptake by the GI tract (68;85;98-100). M-cells concentrated in PP regions are antigen sampling cells of the FAE within the terminal aspect of the gut-associated lymphoid tissue (GALT) and are implicated in active transcytotic processing of environmental antigens for mucosal immunity (85;98;101). M-cells have low metabolic activity and trancytose particles without modification, providing a viable route for macromolecular and colloidal particle absorption (32;76;85;102-104). The epithelium covering the dome region consists of cuboidal epithelial cells and few goblet cells which limits mucus secretion in these areas (101). Hypothetically, molecular binding agents attached to the surface of a particulate delivery system having an affinity to M-cells should increase the amount of carrier and its contents localized in this region, and in the case of it also being a hapten, induce the immune response (102;105-110). Site-specific uptake and internalization of colloids by PPs have been demonstrated in the mouse, rat, and rabbit (98;103;104;106;108;111-118). Quantitative uptake of polystyrene latex beads (600-750 nm) in rabbit intestinal loops confirmed their specific uptake by M-cells, not villous cells, and uptake occurred rapidly (within 10 min) in a synchronous pattern (119). Furthermore, monoclonal anti-M-cell antibodies attached to polymeric microspheres (~1 μm) enhanced binding and internalization by 3-3.5-fold (120). In comparison, liposomes containing covalently-linked IgA (~400 nm) enhanced the local secretory immune response to encapsulated ferritin due to an increase in liposome uptake by M-cells (121). Tomizawa et al. (122) demonstrated a size- and charge- dependency of liposome uptake after intestinal administration of negatively-charged liposomes of EPC:CH:phosphatidylserine (PS) (7:2:3 m.r.) to rats. Liposome encapsulation of

6-carboxyfluorescein (6-CF) promoted its uptake by PPs as well as non-lymphoid tissue. Uptake in PP tissue was shown to be a function of the molar concentration of PS (up to a maximum of 25 percent) in the liposomes. Subsequently, Aramaki et al. (123) demonstrated that DSPC:CH:PS (7:2:3 m.r.) liposomes larger than 374 nm were not only stable under acidic conditions, in diluted bile salt and pancreatin solutions but were preferentially absorbed by PP tissue in the lower ileum. Thus, PS was advocated as an integral liposome component for successful antigen delivery to the GALT. M-cell endocytosis and transcytosis of targeted liposomes was further substantiated by Childers et al. (124) in the rat model using SUVs (<200 nm) as well as gold-core SUVs (~600 nm). Some detailed studies of the surface of an M-cell have shown a thin ~20 nm glycocalyx covering which retarded access of CTB-microparticles greater than 1 μm to the $G_{M1}$ glycolipid receptor on the cell surface (125).

Interspecies variability of PPs, M-cell distribution, and glycoconjugate surface expression account for the wide range in total amounts delivered and for differences in the site-specific accumulation observed (98;103;111;116;126;127). In human, site-specific uptake by PPs appears to be limited and age-dependent, PP prevalence being highest in the fetus and slowly diminishing over time, becoming of limited importance in the elderly (85). The occurrence and activity of PPs are also influenced by diet and extent of bacterial colonization which varies widely in the human population (128). The inconsistent occurrence of PPs may make it difficult to achieve reproducible oral delivery in humans. Nevertheless, M-cell-directed oral vaccination is becoming increasingly relevant since potent immunogens can be delivered directly to the GALT (108;129). Vaccines are intended to stimulate IgA precursor cells which migrate through the common mucosal immune system into the lymphatic and general circulations, ultimately resulting in localization in mucosal tissues where secretory IgA is produced (129;130).

### *1.4.2 Factors Affecting Lymphatic and Systemic Disposition*

This section attempts to clarify issues relating to factors influencing systemic or lymphatic disposition of colloidal delivery systems, including liposomes, after oral administration. The intention of discussing these issues is to illustrate the relationship between experimental models, the sampling site (i.e. lymph or blood), and the conclusions drawn which has bearing on successful interpretation of the scientific evidence presented to date.

The endothelial cells of the capillaries are joined by tight junctions, but contain diaphragm-covered fenestrations (60-100 nm in diameter), allowing slow diffusion of macromolecules into the circulation. Depending on the rate of delivery, a protein, for example, may overflow into the lymphatic system by permeation through endothelial linings located centrally in the lamina propria (30). On examination of orally-administered OVA (50mg OVA, MW ~45000), 80 percent of the absorbed protein was transferred directly to the systemic circulation yielding a lymph:plasma ratio (LPR) of 0.24:1. Reducing the dose by 80 percent (10mg) yielded a LPR of 0.094:1, a result which may be explained by less OVA overflow from the systemic circulation coupled with low lymph flow-rates. In liposomes, the oral bioavailability of OVA doubled exhibiting a sustained absorption profile (30). The relative roles of stability and protection of OVA in liposomes versus extended M-cell processing were not taken into account. Prolonged systemic uptake after intraduodenal administration of solid lipid nanospheres (SLN, ~110-140 nm) to rats has also been reported, with 0.2 and 1.4 percent/g found in blood and lymph at 45 min, respectively, with no change in particle size distribution after absorption (131). Another finding was a reduction in transepithelial passage of 2 μm polystyrene microcapsules in diabetic rats after oral administration compared to healthy controls (132). In this case, it was thought that GI motility played a role in the absorption process because of its effect on residence times at absorption sites. Alternatively, total parenteral nutrition (TPN) increased the uptake of latex particles (~3.2 μm) 5-fold possibly due to the effects of TPN on reducing secretion of mucus and mucosal antibodies, although the effect of TPN on increasing lymphatic drainage (i.e. increasing lymphatic flow) was not considered as a potential factor in particle translocation (133). Lymphatic uptake of 1 μm polystyrene microspheres was also related to age and dose (134), more particles being absorbed in older rats even though the prevalence of PPs is generally found greater in the young (76;85), and re-affirming that the rate of uptake by the GI epithelia determines the overall LPR (30). LeFevre et al. (135) argued that GI transit-time differences and higher clearance rates from PP sites in younger animals were responsible for the reduced particle uptake compared to older rats. Perhaps, increased uptake in older animals may even be a result of greater membrane permeabilities inherent in aged epithelial surfaces. Lecithin-coated polystyrene microspheres (0.5 μm) underwent increased lymphatic delivery whereas oleic acid-coated underwent decreased lymphatic delivery (136), thus demonstrating that the nature of the surface coating plays a role in selection of the disposition pathway.

Evidence of liposome absorption from the GI tract is inconclusive at best, and may be partly confounded by the characteristics of the sampling sites. Predilection of colloidal particles for the systemic or lymphatic circulation depends on the animal species tested and its state of health, the developmental stages of circulatory and lymphatic architectures, the intestinal cell-type targeted, and the formulation administered. The circulatory and lymphatic networks have a non-exclusive symbiotic relationship which is not generally acknowledged in drug delivery, hence the physiological consequences of delivering high-molecular weight proteins, conjugates, and colloids to intestinal sub-epithelial capillaries remain unclear. It has been consistently found that lymphatic uptake of macromolecules favors lipophilicity, high molecular weight, or large molecular size (100). Since lymph flow is much slower than blood flow (1/200 - 1/500), lymphatic uptake and delivery may only be significant for delivery systems that have selective affinity for the FAE. Although lymphatic routing allows circumvention of first-pass metabolism, localization of drug in M-cells, especially a protein and carrier, may inadvertently stimulate an immune response precluding exploitation of the lymphatic route by routine oral delivery.

### 1.5. Strategies

Considering the evidence presented in Sections 1.2 and 1.3, the role of liposomes for improving oral therapies is seemingly tenable, although the variability in PK and PD responses reported throughout the literature suggests that it is encumbent upon formulators to better characterize liposome systems in terms of measurable *in vivo* performance before useful human dosage forms can be generated. With this in mind, strategies concerning liposome stability and GI affinity have been forwarded.

### *1.5.1 Stability*

Undoubtedly, a liposome formulation that retains a therapeutic amount of its payload after oral administration, until it reaches the small intestine is required if any measure of success is to be obtained. Variations in liposome composition alone have not yielded the level of stability required although lipid compositions of DPPC:CH or DSPC:CH (7:2 m.r.) were reported to have high levels of stability (2;13). Recently, substitution of CH with a soybean-derived sterol mixture (DPPC:SS 7:4 m.r.) further increased liposome stability (40;41). The three destabilizing influences encountered by an oral liposome are pH, bile salts, and pancreatic enzymes. As the pH swings from 1-2 in the fasting stomach to 6.5-7 in the proximal intestine the ionization state of the phospholipids, charged adducts, and entrapped solutes can also change and often one of the solute species permeates the bilayer faster than the other. Liposomes must also be able to resist solubilization in approximately a 10 mM bile salt solution which is more likely with MLVs than SUVs or large unilamellar vesicles (LUVs) since a critical solubilizing ratio, $R_e^c$, must be obtained (137). Mixed micelles of phospholipid and bile salts could fall below the $R_e^c$ after the first few lamellae are solubilized (a function of liposome concentration) allowing the remaining intact liposomes to advance to their target absorption site. There are several reports, however, that attest to the instability of liposomes in bile salt solutions (13;138-143), particularly fluid-state liposomes (i.e. at temperatures > $T_m$ of the PC) (142). Phospholipases and other digestive enzymes, if given enough time in the presence of the 'surface-friendly' liposome, will hydrolyze phospholipids, disrupting liposome integrity and resulting in leakage. Charged or sterically-hindered ('surface-unfriendly') liposomes appear to have a greater chance of survival under these conditions. With this in mind, strategies to alter the liposome surface, or at least reduce exposure of the liposome to the environment, have been advanced.

The notion of a polymerized liposome administered orally has attracted limited attention since problems of liposome digestion after absorption, and possible toxicities associated with free radicals retained from processing have been lingering concerns. The preparation of polymerized liposomes has been described by Regen (144). Okada et al. (145) found that retention of $^{14}$C-bovine serum albumin (BSA) (or $^{14}$C-sucrose) in polymerized liposomes after 6 days under simulated GI fluid conditions was about 70 percent compared to 30 percent from regular liposomes. Also, using polymerized radiolabelled liposomes Chen et al. (102) demonstrated liposome integrity in the GI tract, transport of calcein in macrophage cultures, and release of encapsulated materials, resulting in 2.7 percent of the administered dose being transported into the systemic circulation.

The coupling of polymers and liposomes is not a new idea but using the strategy of shielding the liposome and its contents from the hostile environment of the GI tract by its encapsulation in a polymeric shell would appear to have some interesting possibilities. Microencapsulated liposomes have been formulated in different ways. An early prototype was developed by Regen et al. (146) referred to as 'polymer-encased liposomes' in which liposomes were ionically encased within two concentric poly(methacrylate) monolayers, thereby increasing the stability of the liposome. An interesting variation of this utilizes liposomes prepared from amphiphiles which possess a polymerizable group linked to the polar head group by a cleavable spacer followed by cleavage of the spacer after polymerization and resulting in a liposome suspended within a microcapsule (147). Ringsdorf and co-workers (147;148) applied the autopolymerization of 4-vinylpyridine after salt formation with a negatively-charged component (PS, phosphatidic acid (PA), or DCP) at the liposome surface. In this way, the liposome became essentially shrouded by a mesh-like network of polymeric strands ('liposomes in a net'). Perhaps, more of a 'pseudo' polymeric shell was created which offered some protection of the liposome against enzymatic macromolecules, but offers little resistance to the invasion of smaller molecules such as detergents and small ions. When the stability of DMPC:CH:DCP liposomes surrounded by a net of poly(4-vinylpyridine) was tested (149) a significant but low resistance of the liposome to disruption by sodium cholate or isopropanol solution was observed. Yeung and Nixon (150) prepared liposomes microencapsulated by a nylon shell or a combination of nylon and gelatin or gelatin-acacia employing a coacervation technique (151). Drug released from the microcapsules was retarded, being controlled by diffusion from the vesicles, however the liposomes were unprotected when dispersed in aqueous isopropyl alcohol. In 1980, Sun et al. (152) reported a method of microencapsulating live insulin-producing cells by polylysine without exposing the cells to organic solvent thereby maintaining their integrity. In similar fashion, Kibat et al. (153) encapsulated liposomes of hydrogenated soy lecithin or PC:CH (1:1 m.r.) containing FITC-BSA or FITC-HRP within a wall of poly(L-lysine) (154). Although not advocated for oral delivery, conceivably such a system might deliver smaller molecules, for example insulin, to the GI mucosa whereby the rate of drug released from the microcapsules is controlled by release from the liposomes within. Also, such microcapsules incorporated in a tablet or capsule could provide sustained release but have limitations in targeted oral drug delivery. Acacia-gelatin microencapsulated DPPC liposomes prepared by complex coacervation decreased the release of acetylsalicylic acid (ASA) in sodium cholate solutions and was dependent on the concentration of acacia-gelatin, the hardening time using formaldehyde, pH, and the temperature (155). The biodegradable nature of the wall material is desirable and could be further developed to target intestinal sites.

A more simplified approach to stabilizing liposomes with polymer is to allow a water-soluble polymer to adsorb at the liposome surface. Although endogenous macromolecules, such as proteins, interact with particles in this manner, the deliberate insertion of an anchor group attached to the polymer in the bilayers of liposomes is required to retain its film properties or coating of the liposome. Sunamoto and co-workers (156) prepared a series of hydrophobic anchors of palmitoyl chains or cholesterol attached to polysaccharides, including dextran, pullulan, mannan, and amylopectin and coated liposomes on the premise that this would mimic a bacterial or plant cell membrane. The resultant liposomes were found to have increased stability against enzymatic lysis and lower leakage of carboxyfluorescein and $^3$H-inulin (157). An artificial red blood cell has also been prepared by adsorbing carboxymethyl chitin (CM-chitin) on hemoglobin-loaded liposomes (158). These were stable upon exposure to globulin, fibrinogen, and albumin but not in the presence of surfactants (159). Alternatively, a method of preparing a polymer-coated liposome with a pH-sensitive poly(carboxylic acid) derivative has been reported (160;161). In particular, poly($\alpha$-ethylacrylic acid)-coated liposomes were stabilized at pH 7.4 but fluidized at pH 6.8. This could be modulated by changes in the chemical structure and the tacticity of the polyacid (162;163). The preparation of poly(acrylic acid)-complexed DPPC liposomes and release of glucose has been described (164). Increased glucose concentration and ionic strength exerted similar effects of decreased release kinetics but the specific role of the polymer coating is unclear because there was no comparison made to uncoated liposomes. Among these approaches adsorbed polysaccharides would appear to be more advantageous than poly(carboxylic acid)-coated liposomes (assuming similar bioadhesive properties) when the formulation is intended for oral delivery of drug mainly because the solubilizing ability of the polymer at physiologic pH would result in loss of entrapped agent. CM-chitin, O-palmitoyl pullulan (OPP), cholesterol-derivatized pullulan (CHP), and cholesterol-derivatized mannan (CHM)-coated liposomes have been prepared and subsequent leakage of ASA, cytarabine arabinoside (Ara-C), and insulin measured. ASA leakage was retarded 3-fold from CM-chitin-coated DPPC liposomes at pH 5.6, 37°C (165), Ara-C leakage was retarded from OPP-coated DPPC:CH:DCP liposomes at pH 2.0 and pH 7.4 in the presence of 10 mM sodium cholate (166), and to a greater extent when liposomes were coated with CHP (167). Insulin release from CHM-coated DSPC:CH:DMPG (7:2:0.5 m.r.) liposomes was slowed 3-fold under simulated intestinal fluid conditions (168). O'Connor et al. had claimed that coating EPC liposomes with OPP actually increased leakage in the presence of bile salts. This discrepancy is probably related to the degree of co-operativity of the bilayer molecules which increased as a result of "anchoring" of the polysaccharide (169) to liposomes having a higher $T_m$ than EPC liposomes ($T_m$ = -5°C). It was also expected that extension of the polymeric chains from the liposome surfaces inhibited the lytic potential of liposomes by phospholipases. Chitosan appears to be a functional polysaccharide as a stabilizer of liposomes both in vitro (170) and in vivo (51). Low-deacetylated chitosans had stronger associations with liposomes than high-deacetylated chitosans (170). Proteoliposomes prepared from phospholipids of *Escherichia coli* were stabilized for the short-term with hydrophobized (linear hexadecyl chains) dextrans but long-term in the absence of integral protein in the bilayers (171). Also, hydrophobized water-soluble, synthetic poly(N-isopropylacrylamides) adsorbed and strengthened unilamellar vesicle bilayers and responded reversibly to changes in temperature (172). These preliminary studies provide added expectations that water-soluble polymers, natural or synthetic, can yield the required stability of liposomes for oral administration under the appropriate conditions.

### *1.5.2 Gastro-intestinal Affinity*

Bioadhesion and mucoadhesion are terms used to describe adhesive potential to biological or mucosal substrates, respectively (104;173;174). Adhesion to the mucus barrier of the GI tract generally results in slower transit times of dosage forms (175), but its retention is dependent on the mucus turn-over time which has been estimated to be 1-6 h in human (104;174;176). Bioadhesion to the epithelial surface of the GI tract could potentially result in much longer residence times than mucoadhesive systems. However, the extent of bioadhesion of a particle or macromolecule is subject to its independence of mucus flow. Formulations that possess site-specific moieties that have strong affinities to epithelial cell surfaces increase the opportunities of the formulation being retained at the absorbing surface and eventually processed by one of the routing mechanisms discussed previously. Possible candidates identified for this purpose have been lectins and other carbohydrates of the cell glycocalyx allowing certain cell types of the GI tract to be preferentially targeted. Bioadhesives may induce a cellular response upon adhesion, thereby reducing enzymatic activity, opening tight junctions, or inducing endocytosis, all of which may render a compound more bioavailable.

### *1.5.2.1 Carbohydrates*

In addition to increasing liposome stability in the GI tract, polysaccharide polymer coatings may have increased affinity thereby leading to an improvement in bioavailability of insulin after oral delivery (51). Mannan-coated liposomes improved PP uptake of PS-containing liposomes in rats using an optimal liposome size (416 nm) (122). PP tissue uptake was predominant over non-lymphoid tissue uptake for all formulations tested, and a specific affinity of negatively-charged mannan-coated liposomes to PP tissue was found, based on competitive binding studies. In a recent study the ability of CHM-coated DSPC:CH:DMPG liposomes containing insulin to lower blood glucose levels in diabetic rats after oral administration was demonstrated (177). Glucose levels were not only lowered by about 25 percent below controls after 4 h but the activity was sustained for at least 12 h after a dose of 280 IU/Kg in liposomes, compared to 400 IU/Kg insulin in solution.

### *1.5.2.2 Glycoproteins*

Lectins, glycoproteins of plant, bacterial, or animal origin, bind terminal carbohydrate moieties of the enterocyte glycocalyx and/or the mucous glycoprotein network secreted by intestinal goblet cells. The enterocyte glycocalyx is involved in key cellular processes such as antigen recognition, differentiation, transport, and malignant transformation (102). Dietary lectins and microflora modulate enterocyte differentiation along the GI tract and, consequently, may serve as targeting agents for specific GI sites in histochemical, morphological and cell function investigations, or for drug delivery objectives (98;102;128;174;178). During the course of enterocyte differentiation along the crypt-villus axis, the glycocalyx develops from oligomannose residues forming complex glucosyl side-chains, generally terminating in fucose or sialic acid residues. Glycosylation of the enterocyte surface depends on the species, stage of differentiation, location in the GI tract, age, blood group specificity, diet, and bacterial microflora of the animal (128;178). There is evidence that lectins bind avidly to the GI tract epithelium, being subsequently endocytosed and transcytosed (83;85;98;104;111;174;179;180). It is noted, however, that the toxicity of certain lectins preclude them from use in targeted drug delivery (128;180;181). Lectins belong to a group of ribosome inactivating proteins (RIP), type 1 or 2, existing as single-chain proteins or as active A-chains covalently linked to binding B-chains, respectively (182). Type 2 RIP are systemically absorbed and are generally more toxic than type 1 RIP due to the augmentation of uptake mediated by the B-chains (104;180;181). Some examples include PHA, con A, ricin, abrin, modeccin, volkensin, and viscumin. Other type 2 RIP toxins, such as Pseudomonas aeruginosa exotoxin A, diptheria toxin (DT), tetanus toxin (TT), or CT have similar characteristics, exhibiting no systemic availability or toxicity in the absence of the B-chains (75;179). Thus, type 1 RIP lectins and B-subunits of type 2 RIP lectins may serve as affinity molecules for binding to and uptake of macromolecules and colloids by the GI tract (104). It appears that lectin etiology dictates the cellular response. For example, WGA and tomato lectin (TL) are endocytosed by Caco-2 cultured cells while NAcGal specific lectins, such as mistletoe isolectins I,II, and III, are not (183). In contrast, a previous study involving the transport of TL, phytohemagglutinins $L_4$, and $E_4$ across Caco-2 monolayers showed negligible transcytosis in spite of specific binding, possibly due to non-reactive bioadhesion, apical recyclization (184), or cross-reactivity with mucus layers (185). Lectins having affinities to M-cells or enterocytes, which also induce endocytosis, are stable in the GI tract, and have low toxicities are good candidates as targeting agents for drug delivery (75;104;111). In particular, lectins specific for complex glucosyl side-chains (e.g. fucose or sialic acid terminated residues) appear to have the best potential. Table 1-3 cites lectin-mediated applications that are relevant to oral drug delivery.

#### 1.5.2.2.1 Plant Lectins

Lysopersicon esculentum (TL) was one of the first lectins explored as a candidate in oral drug delivery because of its low toxicity, site-specificity, and stability to intestinal enzymes (104). Polystyrene microparticles labeled with TL had a 3-fold greater intestinal absorption and a 9-12-fold higher systemic absorption compared to unlabeled polystyrene microparticles. When N-acetylchiototetraose (a competitive inhibitor of TL) was incubated with TL-microparticles prior to dosing, the augmentation of bioavailability by TL was nullified (186). The uptake of the TL conjugates by villous tissue was 14 times greater than by PPs indicating a shunting of absorptive sites and correlating with the availability of glycosidic binding sites for TL (112;186-188). This shift in absorption site from lymphoid to non-lymphoid tissue signifies a potential control mechanism for site-specific delivery (187). Latex particles labeled with TL, asparagus pea lectin (AL), or mycoplasma gallisepticum lectin (ML) administered ex vivo to rat intestinal tissue exhibited decreasing intestinal binding from the duodenum to the ileum. Binding of the lectins to the intestine corresponded to the carbohydrate composition of the intestinal glycocalyx being TL > ML = AL. Most notably, TL was not preferentially bound to intestinal M-cells, but ML and AL binding increased 25 and 50 percent, respectively, when perfused through intestine containing lymphoid tissue (i.e. PP tissue) (189). Lectin-bearing polymerized liposome delivery improved up to 3-fold over unlabeled liposomes. WGA-liposomes (selective for sialic acid residues) were absorbed by 5.8 percent having affinity for both M-cells and intestinal enterocytes, whereas UEA-1-liposomes (selective for fucose residues) were absorbed by 10.5 percent due to the specific affinity of UEA-1 for PPs (115). A trial study involving encapsulation of $Fe_3O_4$ in polymerized liposomes rendering them magnetically responsive, showed that longer transit times were possible when a magnetic field was placed in position over the rat mid-section (190). The resultant uptake of liposomes into mouse tissues was calculated to be ~5.6 ± 1 percent.

#### 1.5.2.2.2 Bacterial/viral Lectins

Reovirons, rotavirons, adenovirons, polio virus, *Vibrio cholerae*, *Campylobacter jejuni*, *Streptococcus sanguis*, *Shigella flexneri*, *Salmonella typhi*, *Yersinia enterocolitica*, *mycoplasma sp.*, and *Escherichia coli*, are all known pathogens that are capable of entering the systemic circulation via M-cells (80;85;104;189). Commensal luminal bacteria rarely interact with M-cells, however pathogens and/or their respective toxins may adhere to or invade M-cells selectively, producing undesirable local or systemic responses (85). The reovirus capsid protein (σ1) and RDEC-1 *E.coli* fimbrial proteins are examples of bacterial/viral lectins which mediate site-specific attachment to M-cells and may be useful candidates for application in targeted drug delivery (104;173). Reovirus capsid protein attachment to soy phosphatidylcholine (SPC) liposomes resulted in a 10-fold increase in their attachment to a model cell culture. Furthermore, co-incubation of σ1-coated liposomes with isolated rat PP tissue resulted in a 10-20 fold improvement in uptake, indicating selective adherence of the carrier system to M-cells (191). This compares to the M-cell targeting of polystyrene latex beads labeled with ML (specific for sialic acid residues) (189). Likewise, 1μm polystyrene microspheres labeled with *Yersinia pseudotuberculosis* Invasin protein resulted in their increased uptake and transport across cultured epithelial cell monolayers via RME, dubbed a bioinvasive drug carrier (192).

#### 1.5.2.2.3 Toxins

The B sub-unit of cholera toxin (CTB) specifically binds to ganglioside $G_{M1}$ on the enterocyte surface. Consequently, liposome surface attachment of CTB enhanced their binding to ileal brush border tissue, demonstrating the potential of CTB for site-directed delivery (80;193). M-cell selectivity occurs because of increased exposure of $G_{M1}$ at the cellular surface compared to other cells, possibly related to a reduction in apical M-cell microvilli and a less dense glycocalyx (85;194). Both 15 nm colloidal gold and 1 μm latex microparticles labeled with CTB were selectively transcytosed by rabbit M-cells (194). Reports of RME of CTB and TT implicated a role of non-coated pit (caveolae) involvement for uptake and translocation of the toxin in cultured cells (97). CTB is a potent nontoxic adjuvant and induces long-term immunological memory when administered orally. Thus, it may have many applications in antigen delivery, notwithstanding the potential deleterious immunological consequences with repeated CTB administration (80;130). Alginate microspheres (0.5-5 μm) containing CTB linked with pneumococcal capsular polysaccharide administered orally to mice resulted in both mucosal and systemic antibody titers (195). As mentioned previously, covalent conjugation of CTB and CT to SUVs retained the inherent immunogenicity of the native antigens, retained binding affinity for $G_{M1}$ gangliosides, and resulted in serum IgG and salivary IgA anti-CT responses (196). Although CTB has proven to mediate M-cell-specific attachment, Frey et al. (125) demonstrated a size dependency of intestinal binding by using a series of CTB-probes. CTB-FITC (~6.4 nm) was bound to apical membranes of all intestinal cell-types, CTB-colloidal gold (~28.8 nm) was bound selectively to M-cells, whereas CTB-polystyrene microparticles (~1.13 μm) failed to adhere to any epithelial surface.

#### *1.5.2.3 Nutritional factors*

The potential use of cellular nutrients as mediators of macromolecular and colloidal uptake is appealing since RME is a cellular process designed for transporting critical molecules associated with the viability of a cell (197).

##### 1.5.2.3.1 Cyanocobalamin (vit $B_{12}$)

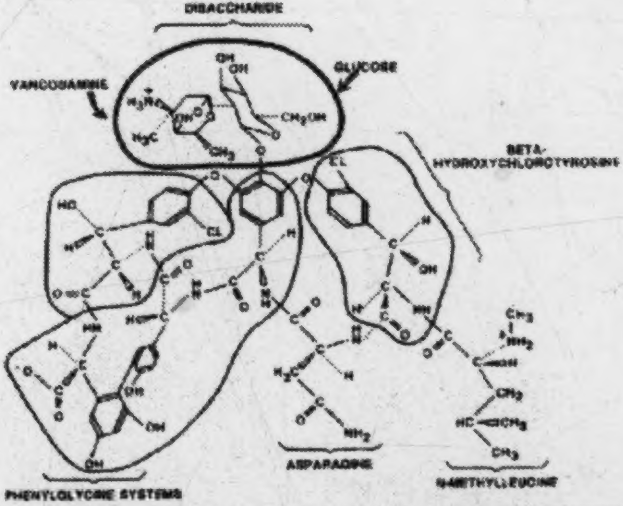
Russell-Jones and coworkers (83) have explored vit $B_{12}$-RME in a variety of applications. Vit $B_{12}$-targeted nano- or microparticles and proteins increased the uptake by this route $10^2$-$10^6$ -fold. In vitro experiments with Caco-2 cell culture recently demonstrated vit $B_{12}$-directed uptake of fluorescent 100 nm nanospheres (198). Uptake was enhanced by addition of intrinsic factor to the medium. Orally-administered fluorescent nanoparticles linked to LTB, con A, or vit $B_{12}$ or administered directly to intestinal loops of rats were found bound to the surfaces of intestinal villous cells, and after a time were found localized within the central lacteals (83). In another study of the oral administration of vit $B_{12}$ nanoparticles to rats, histological examination clearly indicated their accumulation below the mucosal cell layer of villous epithelial cells and eventual traversal to the central lacteal (199).

#### 1.5.2.3.2 Pteroylglutamic (folic) acid

Rapidly-dividing cells express high affinities for FA because folate is an essential factor in purine, nucleotide, and DNA synthesis. Hence, the possibility exists of utilizing this pathway for promoting folate-linked molecules or colloidal particles to enter GI epithelia. FA is absorbed mainly through a saturable, pH dependent, sodium-ion dependent, and metabolic inhibitor-sensitive pathway (200). Folate-mediated uptake of macromolecular conjugates, microparticulates, and liposomes demonstrate the utility and functionality of this routing (87;88;197;201). Folate binding receptors can occur in dense clusters containing up to 700 receptor molecules with estimated densities of ~30,000 molecules/um$^2$, and are GPI linked proteins associated with caveolae (93;202;203). Applicability of folate-drug conjugate RME has been demonstrated for a variety of compounds including doxorubicin, albumin, ribonuclease, horseradish peroxidase, IgG, ferritin, and DNA demonstrating the applicability of folate RME for oncology and gene therapy (88;197;201;204-207). It appears that the nature of the conjugate will determine how the endosomal compartment is eventually routed, for example the highly charged polylysine conjugates are directed towards lysosomal compartments (204;206) and bacterial exotoxins will expediate the release of conjugates into the cytoplasm when desired (201). Folate-liposomes targeted to KB cells (a naso-pharyngeal cell line) have resulted in a 45-fold increase in doxorubicin delivery corresponding to an 86-fold increase in cytotoxicity when compared to non-targeted liposomes (88). As well, through use of an endosomal disrupting peptide (EALA), 20-25% of encapsulated cargo is delivered to the cytoplasm within several hours of administration (87). Cytotoxin-folate conjugates were actively endocytosed by malignant cells in co-culture with normal tissue cells, allowing re-growth of normal tissue (208). The specificity and pH-dependence of folate absorption from the gastrointestinal tract is now well characterized (89). Caco-2 cells express folate receptors and demonstrate pH-dependence of folate uptake; Thus, cytotoxic folate-conjugates were effectively endocytosed to elicit a cellular response (201). To date, reports regarding the oral absorption-promoting effects of FA on associated colloids or drugs for systemic therapy have not been forthcoming.

## 1.6 Vancomycin (VCM)

### *1.6.1. Chemical structure*



Picture adapted from: Cheung, R.P.F. and J.T. DiPiro. 1986. Pharmacother. 6:153-169.

### *1.6.2 Physico-chemical properties*

| | |
|---|---|
| pI (isoelectric point) | 7.0 |
| Charge | $2^+$- $4^-$, pH 4.0 – 12.0 |
| Aqueous solubility | 15 –100 mg $mL^{-1}$ |
| | 15 mg $mL^{-1}$ at pH 7.0 (pI) |
| UV max | 282 ($E^{1\%/1cm}$ 40) |
| MW | 1449.22 |

A solution of VCM hydrochloride is most stable at pH 3-5 and forms a solution of pH ~4 in 0.9 percent NaCl, which remains stable for 2 wk at room temperature (209). The long-term stability of VCM in plasma (>1.5 y) at –20ºC has been reported previously (210). The crystalline degradation product (CDP-1) of VCM, created by hydrolytic conversion of the asparagine residue to isoaspartate, accounts for the major degradation product appearing in buffer solutions and human serum (211).

### *1.6.3 Pharmacokinetic properties*

VCM is a tricyclic glycopeptide antibiotic produced by Streptomyces orientalis and is primarily active against gram-positive organisms (209). Oral VCM therapy, resulting in high local VCM concentrations (1–9 mg $mL^{-1}$) in the distal colon, is indicated for colitis caused by C. difficile infection (209). The oral absorption of VCM is negligible (range 1-4 percent), and is generally independent of bowel and renal status (209,212). VCM is primarily excreted by glomerular filtration, 80-90 percent of the dose appearing unchanged in human urine at 24 h (209). Renal clearance constitutes > 90 percent of total body clearance in rats and remains constant over a large VCM plasma concentration range, averaging 1.5-2.0 mL $min^{-1}$ (213). Thirty –40 percent of VCM excreted in urine is due to tubular secretion likely mediated by the organic cation transport system (213).

## 1.7 Research Proposal

### *1.7.1 Aim*

The overall aim of this research was to develop an orally-administered targeted liposome delivery system for improving the absorption of poorly-absorbed drugs, e.g. proteins/peptides. Vancomycin was used as a model glycopeptide.

### *1.7.2 Hypothesis*

FA-coated liposomes have potential for improving the oral absorption of poorly-absorbable water-soluble solutes, for example proteins or peptides, because of binding of FA to receptors in the small intestine.

### *1.7.3 Objectives*

1. Design and characterize an appropriate liposomal system having optimized VCM encapsulation, and which remains stable in simulated GI environments, including 10 mM bile salts.
2. Design, synthesize, and characterize a hydrophobized FA–linked macromolecular conjugate for insertion in the surface bilayers of liposomes containing VCM.
3. Test uncoated- and coated-liposomal VCM dosage forms for interaction with and tranport across monolayers of cultured Caco-2 intestinal cells.
4. Measure the bioavailability of VCM in FA-targeted liposomes after oral administration to rats.

Table 1-1. Factors influencing oral delivery of drugs in microparticulates.

| |
|---|
| Size |
| Surface characteristics |
| Stability in GI fluids |
| Stability in mucosal epithelium |
| Affinity to localized intestinal epithelium |
| Residence time at the absorption site |
| Encapsulation efficiency |
| Encapsulation mechanism |
| Concentration at the absorption site |

Table 1-2. Evidence of drug absorption from oral liposomes.

| Drug | Dose(route) | Model | Liposome Composition | Evidence of GI uptake and translocation | Ref |
|---|---|---|---|---|---|
| *Insulin* | 12IU<br>60-70mg lipid<br>(po) | Diabetic Rat | EPC:CH:DCP (10:2:1) | 37-59% reduction in BG with liposomes compared to 14.2% reduction for control | (3) |
| | 1.3IU<br>4.2IU<br>15mg lipid (po) | Rat | DPPC:CH (7:2)<br>EPC:CH (7:2) | 23% reduction in BG for DPPC liposomes and no reduction for EPC liposomes | (2) |
| | 1.0IU<br>5.0IU<br>15mg lipid (po) | Diabetic rat | DPPC:CH (7:2)<br>EPC:CH (7:2) | 43% reduction in BG for DPPC liposomes and 23% reduction for EPC liposomes | (2) |
| | 6.0IU<br>(po) | Rat | EPC:CH:DCP (10:2:1) | 46% reduction in BG with co-administration of insulin loaded liposomes and IAA | (5) |
| | 0.3-5.0IU<br>(po) | Diabetic rat | EPC:CH<br>DOPC:CH<br>DSPC:CH<br>DPPC:CH<br>DMPC:CH<br>DLPC:CH<br>(7:2) | 34.3, 54.4, 42.4, 54.9, 52.9, 55.3% reduction in BG, respectively | (6) |
| | 8-10IU/100g<br>(IG, buccal) | Rat | DPPC:CH (7:2) | 45% increase in BG for IG administration and 55% reduction in BG for buccal administration | (8) |

|  |  |  |  |  |  |
|---|---|---|---|---|---|
|  | 5IU<br>(IG) | Diabetic rat and rabbit | DPPC:CH:PA (7:2:1) | 6 of 9 rabbits and 13 of 24 rats responded to liposome therapy (~40% reduction of BG) | (9) |
|  | 1.4-1.6 x $10^6$ cpm $^{125}$I-insulin<br>(IG) | Rat | DPPC:CH:DCP (7:2:1)<br>DPPC:CH:DCP(7:7:1) | 60 and 15% of radioactivity associated with liposomes in portal blood, respectively | (29) |
|  | NT | Diabetic rat | EPC:PI (1:1)<br>DPPC:DPPE (1:1) | Hyperinsulinemia recorded for both types of liposomes, however only semi-synthetic lipids resulted in lowering of blood glucose | (50) |
|  | 20IU/kg<br>(po) | Rat | DPPC:SS (7:4) | Insulin bioavailability up to 32% | (42) |
|  | 24IU<br>(IG) | Rat | DPPC:DCP(8:2)- chitosan coated | 25-30% reduction in BG with prolongation of effect(>12h) | (51) |
| *Inulin* | 20mg lipid<br>(II) | Perfused rabbit ileum | DSPC:CH<br>DSPC:CH:SA<br>DSPC:CH:DCP<br>DPPC:CH:DCP<br>DMPC:CH:DCP<br>SPC:CH:DCP<br>EPC:CH:DCP<br>Free inulin<br>(7:2:1 PC:CH:DCP) | 9,27,31,19,15,6,2,22% uptake of inulin into venous effluent, respectively | (22) |

| | | | | | |
|---|---|---|---|---|---|
| *PEG-4000*<br>*HC*<br>*SA* | NR<br>(IG) | Rat | DSPC:CH MLV<br>DSPC:CH LUV<br>EPC:CH MLV<br>(2:1 PC:CH ) | Liposomes do not provide enhanced uptake of model drugs | (26) |
| *CT and PTH* | NR<br>(IG) | Rat | EPC:SA (9:1)<br>EPC:CH:SA (7:2:1) | 14% and 20% improvement in CT and PTH therapy, respectively | (55) |
| *EPO* | 17928 IU/kg | Rat | DPPC:SS (7:2) | 0.74-31% bioavailability of EPO | (39) |
| *SPK* | 2mg/mL<br>(po) | Cat | HSPC:CH:SA (6:4:1) | liposomal SPK more effective than free SPK | (58) |
| *Factor VIII* | 800 IU<br>(po) | Human | EPC:PA (1:0.05, %w/w) | Lag-time to effect and prolonged F VIII activity when compared to IV therapy | (10) |
| | 1200 IU<br>(po) | Human | EPC:PA (1:0.065, %w/w) | Shortening of bleeding time | (61) |
| | 1000-1200 IU<br>(po) | Human | NT | Variable increase of F VIII:C between 2-23% upon liposomal administration | (62) |
| *Factor IX* | NT | Dog | NT | Rapid absorption of F IX, F II, and F X upon lipo-F IX administration | (62) |
| *Heparin* | 150000units<br>(po) | Dog | EPC:SA (1:0.05) | Increase of a-PTT times with liposomal delivery | (11) |
| *AZT-CDS* | 50mg/kg<br>(IJ) | Rat | EPC:CH (10:2) | Liposome encapsulation resulted in ~5-fold increase in AZT levels compared to a DMSO formulation | (37) |
| *HEPC* | 20mg/kg<br>(IG) | Rat | HEPC:CH:DMPG (4:5:1) | 36.6% increase in AUC for HEPC administered as SUVs | (44) |

| | | | | | |
|---|---|---|---|---|---|
| *SOD* | 0.5-20.0 mg/kg (po) | Rat | DSPC:CH:SA (14:7:4) with and without ceramides | 52-57% relative bioavailability | (46) |
| | 0.5-20.0 mg/kg<br>(po) | Rat | DSPC:CH:SA (14:7:4) with and without ceramides | Dose-dependent protection against carageenan induced paw edema due to reduction in cellular $PGE_2$- and $TXA_2$-synthase activity | (48) |
| *OVA* | 10mg<br>(po) | Rat | EPC:CH:DCP (10:10:1) | 2.3-fold increase in OVA bioavailability | (30) |
| *URIC* | NR<br>(po) | Chicken | EPC:CH:DCP (7:2:1) | 50% fall in BUN, and increase in plasma uricolytic activity | (63) |
| *MOR* & *FEN* | 10mg/kg<br>40Og/kg<br>(po) | Rat | NT | Improved biological effect with liposome delivery | (64) |
| *THBP* | 5mg/kg<br>(po) | Guinea pig | DSPC:CH:DCP (10:10:1) | 50% increase in $C_{max}$ at 1h post-liposome administration | (35) |
| *α-toc* | 3.4-15 mg/kg<br>(IG) | Rat and bile-obstructed rat | EPC<br>EPC:PA<br>DPPC<br>DPPC:PA<br>DSPC<br>DSPC:PA | *α*-toc absorption improves with higher $T_m$ lipids and PA. EPC gave best results for bile-obstructed rats | (32) |
| *ALB* | 50mg/kg<br>(po) | Rat | NR | Enhanced bioavailability of ALB via liposome delivery | (65) |

| | | | | | |
|---|---|---|---|---|---|
| *GFN* | 7mg/kg (po) | Rabbit | EPC:CH:DCP (1:1.6:0.2) | 2.6-fold increase in $C_{max}$, 3.3-fold increase in AUC, 2.1-fold increase in $t_{1/2}$ for GFN-loaded liposomes | (36) |
| *No drug* | NR (IG) | Mouse | dialkyl-PC<br>diether-PC<br>diester-PC | Stabilization of lipid backbone does not improve oral delivery of liposomes | (12) |
| | ~$10^{12}$ particles (IG) | Mouse | polymerized DODPC | 2.71% systemic absorption | (102) |
| | ~$10^{12}$ particles (IG) | Mouse | polymerized DODPC-lectin coated liposomes | 5.8% and 10.5% bioavailability for WGA and UEA-I coated liposomes, respectively | (115) |
| | ~$10^{12}$ particles (IG) | Mouse | polymerized DODPC-$Fe_3O_4$ magnetic liposomes | 60% improvement in systemic uptake upon using a magnetic field (~5.6%) | (190) |

ALB-albendazole, α-toc- α-tocopherol, AZT-CDS- zidovudine chemical delivery system, BER-berberine bisulfate, BG-blood glucose, CT-calcitonin, EGPL-effervescent granule pro-liposome, EPO-erythropoietin, FEN-fentanyl, GFN-griseofulvin, HC-hydrocortisone, IAA-indole acetic acid, INDO-Indomethacin, MOR-morphine, NR-not reported, NT-not translated, OVA-ovalbumin, PTH-parathyroid hormone, SA-salicylic acid, SOD-superoxide dismutase, SPK-streptokinase, THBP-tetrahydrobiopterin, URIC-uricase

Table 1-3. Lectin interactions with the gastrointestinal tract.

| Lectin | Model | Lectin-GIT Physiology | Ref |
|---|---|---|---|
| Panel of 8 lectins | Rat | Lectins and microflora composition induce changes in glycosylation state of lumenal membranes and cytoplasmic glycoconjugates of epithelial cells. | (128) |
| Panel of 5 lectins | Rat | Significant changes in lectin binding patterns due to diet and microflora composition. | (102) |
| Panel of 21 lectins | Porcine jejunum and ileum | Age, region, and cell-type related changes in epithelial glycocalyx and goblet cell mucins. | (178) |
| **Lectin** | **Model** | **Lectin-GIT Specificity** | **Ref** |
| TL, PE, PL | Caco-2 | Affinity rank: PE>PL~TL | (184) |
| | | Lectins show strong bioadhesive nature, however no significant transcytosis | |
| YT | Caco-2 | Increased transport via receptor mediated endocytosis | (192) |
| WGA, TL, MSL I,II,III | Caco-2 | TL and WGA are endocytosed but the NAcGal specific MSL I,II & III are not. | (183) |
| UEA-I | Mouse | Selective for M-cells and is rapidly transcytosed | (111) |
| Panel of 13 lectins | Mouse | Differential binding of EE, BSI-$B_4$, UEA-I, UEA-II to M-cells in PPs of mouse, however lack of differential binding in rat and rabbit. | (126) |
| Panel of 27 lectins | Mouse and Human PPs | Mouse M-cells stained by fucose-specific lectins (UEA-I, AAA), but no differential staining in human FAE | (127) |
| WGA, UEA-1 | Mouse | 2- and 3-fold enhanced uptake for WGA and UEA-1 coated liposomes, respectively. 5.8% uptake for WGA-liposomes and 10.5% uptake for UEA-1-liposomes. | (115) |

|  |  |  |  |
|---|---|---|---|
| TL | Everted gut sacs, Mouse | Resistant to digestion, non-toxic, delays GI transit time, endocytosed and transcytosed, good candidate for macromolecular drug delivery | (81;188;214-218) |
| TL | Rat | TL enhanced uptake of PS microspheres by 3-fold and systemic delivery was enhanced by 9-12-fold. Shunting of absorptive sites to non-lymphoid tissue. | (186) |
| Reovirus σ-1capsid protein | Rat | 10-20 fold increase in liposome uptake upon incubation with rat PPs | (191) |
| CTB | Porcine brush border | CTB liposomes targets gangliosides($G_{M1}$) on epithelial cell surface | (193) |
| CTB | Rabbit | Selective transcytosis of CTB coated colloidal gold and PS microparticles by M-cells | (194) |
| SNA | Human | Selective binding to the FAE | (98) |
| RDEC-I E-coli fimbrial protein | Human | Selective for M-cells and PP uptake | (104;173) |

AAA-aleuria aurentia, AL- asparagus pea lectin, BSI-$B_4$-bandeiraea simplicifolia I isolectin $B_4$, CTB-cholera toxin B sub-unit, EE-euonymus europaeus, ML-mycoplasma gallisepticum lectin, MSL I,II & III-mistletoe lectins, PE-phytohemagglutinin $E_4$, PL-phytohemagglutinin $L_4$, SNA-sambucus nigra, TL-tomato lectin, UEA-I&II- ulex europaeus I&II, WGA-wheat germ agglutinin, YT-yersinia pseudotuberculosis invasin protein

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## *Chapter 2*

# AN INTERFACIAL TENSION MODEL OF THE INTERACTION OF WATER-SOLUBLE POLYMERS WITH PHOSPHOLIPID COMPOSITE MONOLAYERS

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## 2.1 Introduction

An understanding of the role of lipid interactions in the bilayer of vesicular physical systems allows investigators to elucidate mechanisms regarding the stabilization of the lipid membranes. The following describes the development of a simple imaging technique, axisymmetric drop shape analysis (ADSA), for describing the effects of surface-active polymeric additives on the behavior of lipid bilayers.

## 2.2 Background

The air/water and oil/water interfaces have been applied to study the characteristics of biological membranes in the presence of many water-soluble additives, including macromolecules. Although the air/water interface has been most widely investigated, because of its experimental simplicity, it is generally conceded that the oil/water interface would represent a superior physical model of a bilayer by examining the behavior of one-half of a bilayer, i.e. a monolayer in this environment (1). At the liquid/hydrocarbon interface films of biological molecules, such as phospholipids, are presumed to be in an expanded state due to assimilation of their hydrocarbon chains with the bulk liquid hydrocarbon. However, this may depend on the nature of the oil used and the composition of the lipids in the interfacial film. In many situations phospholipid monolayers become exposed to polymers, such as proteins, polysaccharides, or synthetic derivatives. Hence, the nature of the interaction of water-soluble polymers with phospholipid films at the oil/water interface would be of interest. It was anticipated that measurements of interfacial pressures of polymers combined with lipids that often comprise a model biological membrane could help to elucidate these types of interactions and that the relative behavior of the polymers could be assessed.

This study employed a rapid and convenient interfacial tension method previously described (2) for measurements at the oil/water interface in which solutes in both the oil and aqueous phases co-adsorb to produce mixed films. Polymers that are being considered for the formulation of polymer-coated liposomes as an improved liposomal drug delivery system, in particular, have been selected.

## 2.3 Materials and Methods

### *2.3.1 Materials*

Dimyristoylphosphatidylcholine (DMPC) and dipalmitoylphosphatidylcholine (DPPC) were obtained from Princeton Lipids (Princeton, N.J.). Dicetylphosphate (DCP), cholesterol (CH), pullulan (nominal M.W. 54,000), dextran (nominal M.W. 73,500), and polyvinylalcohol (PVA, nominal M.W. 40,000) were obtained from Sigma Chemical Co. (St. Louis, MO) and used as received. Polyacrylic acid (PAA, nominal M.W. 250,000), chlorobenzene (HPLC grade), benzyl benzoate, and chloroform were obtained from Aldrich Chemical Co. (Toronto, ON). Cholesterol-derivitized pullulan and dextran were synthesized and purified by previously published methods (3,4). The purity of solvents was determined from surface tension measurements and comparison to literature values. All water used in the preparation of solutions was double glass-distilled.

### *2.3.2 Interfacial tension measurements*

Interfacial tension measurements were performed using the axi-symmetric drop shape analysis - pendant drop technique (ADSA-P) (5,6). Drops of oil phase were formed from a cleaned, 26 gauge Teflon cannula immersed in water or aqueous solution in a 20mL quartz glass cuvette at ambient temperature (24±1°C). Drop profile images were collected and digitized over 15 min and shape analysis was carried out using a Sun Sparc 10 work station which generates spline curve fits to the drop profile coordinates. The accuracy of the technique is of the order of ±0.1 mN/m (2). The averages of at least six measurements of each drop were determined. Equilibrium at the interface was obtained after 10 min as determined by kinetics of interfacial tension observations as a function of time.

When polymers were used, 1 %w/v aqueous solutions were equilibrated with pendant drop chlorobenzene solutions of lipids. The polymer concentration, although somewhat arbitrary, was selected with reference to the use of polymers to stabilize liposomes and to maintain a relatively low viscosity of the solution.

### *2.3.3 Density measurements*

Densities of solutions, required for interfacial tension calculations, were measured at ambient temperature (24±1°C) using a PAAR DMA 55 densitometer.

## 2.4 Results and Discussion

Figure 2-1 illustrates the variation of interfacial tension at three oil/water interfaces as a function of DMPC concentration on a log scale. In each case the interfacial tension was independent of DMPC concentration up to about $10^{-3}$ to $10^{-2}$ mM then the interfacial tension dropped rapidly up to about $10^{-1}$ mM after which it remained fairly constant, indicative of the formation of a packed monolayer at the interface. Chlorobenzene was selected for further studies because adsorption equilibrium of phospholipid was attained faster than with chloroform, and interpretation of interfacial tension data was expected to be simpler than at the chloroform:benzyl benzoate/water interface. Also, as seen in Figure 2-1, the concentration of DMPC at which a decrease in the interfacial tension occurred was lower at the chlorobenzene:water interface than at the chloroform:water interface. Experimentally, an organic liquid of greater density than water was convenient to use ($d_{20}$ (chlorobenzene) = 1.10 g/cc).

The interfacial tensions of DMPC or DPPC monolayers containing CH and DCP formed at the oil/water interface from chlorobenzene solutions over four orders of magnitude of concentration are graphically represented in Figure 2-2. The shapes of the curves are approximately the same in each case, however, in general, it can be seen that mixtures of DMPC:CH (3:1 mole ratio) or DMPC:CH:DCP (3:1:0.5 mole ratio) yielded higher interfacial tensions than DMPC alone whereas comparable mixtures with DPPC yielded lower interfacial tensions than DPPC alone.

Table 2-1 lists the interfacial tensions ($\gamma_{12}$) and corresponding surface pressures ($\pi$) of lipid composites and polymers at the chlorobenzene:water interface where

$$\pi = \gamma_o - \gamma_{12} \qquad (1)$$

and $\gamma_o$ is the interfacial tension at the clean chlorobenzene/water interface. It is apparent that both phospholipids are highly surface active at this interface but DMPC yielded a higher $\pi$ than DPPC. The addition of CH to DMPC yielded a lower $\pi$, in contrast to the addition of CH to DPPC which yielded a higher $\pi$. Increasing the CH content from 25 to 50 mole percent modestly increased and decreased $\pi$ of DMPC and DPPC monolayers, respectively. In typical plots of $\pi$ versus A (area per molecule) at a given concentration of adsorbate, it is known that curves having higher values of $\pi$ represent films having more expanded characteristics (7-9). Conversely, curves having lower values of $\pi$ represent films in a more condensed state. Thus, the addition of CH resulted in a more condensed DMPC film but a more expanded DPPC film, a finding that is consistent with the effect of CH on the fluidity of phospholipid bilayers above and below the phase transition temperature ($T_m$) of the phospholipid (10) but not in agreement with mixed films of CH and phospholipid at the $CCl_4$ and heptane-water interfaces, at which CH and phospholipid behaved ideally (8). This is in contrast to the air/water interface at which CH condenses spread-films of phospholipid (11). The addition of DCP further condensed both the DMPC:CH and DPPC:CH films (i.e. DCP slightly reduced the degree of expansion of the DPPC due to CH). In comparison, the $\pi$ of adsorbed polymers at the chlorobenzene/water interface was low, except for PVA which has a substantial surface activity as seen by its $\gamma_{12}$ in Table 2-1.

Relative surface pressure ($\pi^*$) was studied in order to investigate the role of monolayer composition on polymer interaction at an oil/water interface and is defined as:

$$\pi^* = \pi_{\text{lipid/polymer}} - \pi_{\text{lipid}} \qquad (2)$$

where $\pi_{\text{lipid}}$ is the surface pressure of the monolayer without polymer and $\pi_{\text{lipid/polymer}}$ is the surface pressure of the lipid monolayer and adsorbed polymer at equilibrium at the chlorobenzene/water interface. A positive $\pi^*$ value is indicative of a polymer that adsorbed at the interface and contributed to an increased surface pressure (film expansion) due to a combination of its affinity for the interface and its effect on the molecules of the lipid monolayer. A negative $\pi^*$ is indicative of a polymer that by virtue of its surface action with the molecules of the lipid monolayer increased the packing of lipid molecules (condensation) at the oil/water interface. Table 2-2 compares the $\pi^*$ values for each of eight water-soluble polymers adsorbed at the interface in conjunction with either DMPC or DPPC. It can be seen that all of the polymers yielded positive values of $\pi^*$ at the DPPC-covered interface and negative values of $\pi^*$ at the DMPC-covered interface. Hence, the film of DMPC underwent condensation as a result of polymer adsorption whereas the film of DPPC became expanded as a result of polymer interaction at the interface.

The modulation of polymer interaction at the phospholipid interface by commonly incorporated lipids such as CH and DCP can be described by a difference expression of $\pi^*$ ($\Delta\pi^*$) given by:

$$\Delta \pi^* = \pi_{II}^* - \pi_I^* \tag{4}$$

where the subscripts II and I refer to the higher and lower lipid composites, respectively. Table 2-3 presents $\Delta\pi^*$ values corresponding to each polymer. The addition of 25 mole percent CH to DMPC films in the presence of 1% w/v polymer clearly resulted in its expansion since $\Delta\pi^*$ is positive and the degree of polymer interaction with the monolayer was similar in all cases (mean $\Delta\pi^*$= 5.77±2.36 mN/m). Increasing the CH content to 50 percent resulted in further expansion of the films (except in the cases of dextran, PAA at pH 6, and PVA). In contrast, the addition of 25 mole percent CH to DPPC films in the presence of polymer resulted in condensation (negative $\Delta\pi^*$), but increasing CH to 50 percent resulted in expansion of the film. For example, CHP condensed the DMPC film by 9.34 mN/m but including CH resulted in expansion by 4.70 mN/m for a net $\pi^*$ of -4.64 mN/m, i.e. an overall condensation of the DMPC film. In comparison, CHP expanded the DPPC film by 3.18 mN/m but including CH resulted in a decrease of 4.86 mN/m for a net $\pi^*$ of -1.68 mN/m, i.e. an overall condensation of the DPPC film also occurred. This compares with the condensation of DMPC film by 5 mN/m and the expansion of DPPC film by 6 mN/m with 25 mole percent CH in the absence of polymer (Table 2-1). The inclusion of 5 mole percent DCP in DMPC:CH films resulted in positive $\Delta\pi^*$ values for each of the polymers (except dextran) indicating expansion, and in the case of CHP, yielding a net $\pi^*$ of -0.8 mN/m. In other words, interfacial pressures of DMPC and DMPC:CH:DCP lipid composite films to which CHP had adsorbed were essentially the same at the chlorobenzene/water interface. This also held for pullulan, dextran, and CHD. There was, however, a net condensation of films of about 3 mN/m containing CM-chitin but a net expansion of films from 4-7 mN/m containing PAA or PVA. The inclusion of 5 mole percent DCP in DPPC:CH films resulted in no significant change in interfacial pressure in the presence of each of the polymers, except when PAA at pH 1.8 or PVA were present in which cases a slight expansion occurred (likely due to the large molecular weight of PAA and the high surface activity of PVA). In the DPPC composite films including CH and DCP the net change in $\pi$ was -1.8 mN/m.

The interaction of a polymer at a lipid interface can be based on both its surface activity and the nature of its interaction with the lipids. Using the $\pi^*$ value of the polymer for a given composite lipid film and the surface pressure of the polymer at the clean interface (Table 2-1), a polymer impact ratio, ($P^*$), was determined from:

$$P^* = \frac{\pi_{\mathrm{lipid/polymer}}}{\pi_{\mathrm{polymer}}}, \qquad (3)$$

where $P^*$ can be utilized to compare polymer performance at different lipid interfaces. A compilation of polymer impact ratios are listed in Table 2-4 for the eight different lipid monolayers. In general, low $P^*$ values indicate a strong interaction and high values a weak interaction with the lipid film. Intermediate values represent attachment but not extensive disruption of the film which would be ideal if surface coatings are desired. Thus, the implication is that strong surface active polymers represented by PVA may be detrimental to biological membranes whereas weakly interacting polymers such as pullulan or dextran would not be beneficial in the formulation of sterically-stabilized liposomes, for example. A $2.0 < P^{*} < 2.5$ obtained for CHP, CHD, CM-chitin, and PAA at pH 6 would appear to be ideal since some of these polymers have also been shown to stabilize liposomes in simulated gastric and intestinal fluids (12,13).

One of the main findings was the opposite effects of mutually-incorporated CH:DCP and polymer on the state of packing of the monolayer, one counteracting the action of the other. This leads to the conclusion that CH and polymer interact at different sites of the monolayer and do not necessarily interfere with each other. Hence, the water-soluble polymers caused changes in monolayer packing mainly in the polar headgroup region, whereas CH altered van der Waals attractive forces between the hydrocarbon chains of the phospholipid even in an environment of chlorobenzene molecules. The monolayer expansion tendencies of negatively-charged DCP also predominated in the hydrophilic region. Thus, the state of condensation or expansion of model or biological membranes is highly dependent on the type of polymer and the composition of the membrane, the parameters of which can be readily evaluated by interfacial tension measurements at the oil/water interface.

Table 2-1. Interfacial tension ($\gamma_{12}$) at the chlorobenzene/water[1] interface and surface pressure ($\pi$) of adsorbed lipid films.

| Lipid Composition[2] (mole ratio) | Interfacial Tension MN/m (s.d., n=6) | Surface Pressure mN/m |
|---|---|---|
| DMPC | 0.68 (0.35) | 35.50 |
| DMPC:CH (3:1) | 6.23 (0.34) | 29.95 |
| DMPC:CH (1:1) | 4.25 (0.75) | 31.93 |
| DMPC:CH:DCP (3:1:0.5) | 7.99 (0.61) | 28.19 |
| DPPC | 6.59 (2.11) | 29.59 |
| DPPC:CH (3:1) | 0.80 (0.48) | 35.38 |
| DPPC:CH (1:1) | 4.10 (0.77) | 32.08 |
| DPPC:CH:DCP (3:1:0.5) | 1.35 (0.45) | 34.83 |
| CHP | 22.12 (2.07) | 14.06 |
| Pullulan | 28.82 (0.42) | 7.36 |
| CHD | 22.69 (0.93) | 13.49 |
| Dextran | 29.38 (2.66) | 6.80 |
| CM-Chitin | 21.21 (0.48) | 14.97 |
| PAA (pH=6) | 21.90 (0.28) | 14.28 |
| PAA (pH=1.8) | 20.05 (1.04) | 16.13 |
| PVA | 4.16 (0.98) | 32.02 |

[1] $\gamma_{12}$ = 36.48 mN/M.

[2] [DMPC] and [DPPC] was 0.1 mM; [PC:CH] was 0.133 mM; [PC:CH:DCP] was 0.150 mM.; [polymer] was 1% w/v.

Table 2-2. Relative surface pressure values ($\pi^{*}$)[1] of polymers at the chlorobenzene/water interface containing films of two lipid compositions.

| Lipid Composition[2] | CHP | Pullulan | CHD | Dextran | CM-Chitin | PAA (pH=6) | PAA (pH=1.8) | PVA |
|---|---|---|---|---|---|---|---|---|
| DMPC | -9.34<br>(0.10) | -9.39<br>(0.24) | -9.70<br>(0.28) | -8.07<br>(0.05) | -6.66<br>(0.23) | -4.08<br>(0.67) | -10.09<br>(0.08) | -2.36<br>(0.58) |
| DPPC | 3.18<br>(0.33) | 3.05<br>(0.50) | 3.43<br>(0.56) | 3.49<br>(0.51) | 4.99<br>(0.23) | 6.15<br>(0.17) | 5.74<br>(0.31) | 4.47<br>(0.60) |

[1] mean (± s.d., n=6, mN/m)

[2] [PC] was 0.1 mM.

Table 2-3. Differential Effects of CH and DCP on relative surface pressure ($\Delta\pi^{*}$)[1] of lipid films.

| Lipid Composition | CHP | Pullulan | CHD | Dextran | CM-Chitin | PAA (pH=6) | PAA (pH=1.8) | PVA |
|---|---|---|---|---|---|---|---|---|
| DMPC | | | | | | | | |
| CH (3:1) | 4.70 | 3.22 | 4.77 | 8.05 | 3.65 | 5.02 | 10.21 | 6.50 |
| CH (1:1) | 10.70 | 9.65 | 8.40 | 7.97 | 6.87 | 5.67 | 12.97 | 6.04 |
| DPPC | | | | | | | | |
| CH (3:1) | -4.86 | -4.54 | -4.77 | -6.04 | -6.28 | -6.35 | -8.16 | -6.58 |
| CH (1:1) | -1.35 | -1.78 | -2.20 | -2.79 | -4.99 | -4.29 | -3.53 | -3.62 |
| DMPC:CH | | | | | | | | |
| DCP (3:1:0.5) | 3.88 | 4.62 | 4.79 | 0.02 | 5.93 | 6.26 | 3.85 | 3.22 |
| DPPC:CH | | | | | | | | |
| DCP (3:1:0.5) | -0.02 | -0.20 | 0.66 | 0.79 | 0.53 | 0.68 | 1.60 | 1.84 |

[1] $\Delta\pi^{*}=\pi^{*}_{1}-\pi^{*}_{o}$

Table 2-4. Polymer impact ratios ($P^*$) indicating relative surface pressure effects of polymer on various lipid films.

| Lipid Composition[1,2] (mole ratio) | CHP | Pullulan | CHD | Dextran | CM-Chitin | PAA (pH=6) | PAA (pH=1.8) | PVA |
|---|---|---|---|---|---|---|---|---|
| A | 1.86 | 3.55 | 1.91 | 4.03 | 1.93 | 2.20 | 1.58 | 1.04 |
| B | 1.80 | 3.23 | 1.86 | 4.40 | 1.80 | 2.16 | 1.86 | 1.06 |
| C | 2.37 | 4.37 | 2.27 | 4.68 | 2.15 | 2.35 | 2.16 | 1.11 |
| D | 1.95 | 3.62 | 2.08 | 4.15 | 2.08 | 2.48 | 1.99 | 1.11 |
| E | 2.33 | 4.43 | 2.45 | 4.87 | 2.31 | 2.50 | 2.19 | 1.06 |
| F | 2.40 | 4.60 | 2.52 | 4.83 | 2.28 | 2.46 | 2.04 | 1.04 |
| G | 2.41 | 4.53 | 2.47 | 4.82 | 2.14 | 2.38 | 2.12 | 1.03 |
| H | 2.36 | 4.50 | 2.53 | 4.86 | 2.28 | 2.47 | 2.11 | 1.08 |

[1] A. DMPC, B. DMPC:CH (3:1), C. DMPC:CH (1:1), D. DMPC:CH:DCP (3:1:0.5), E. DPPC F. DPPC:CH (3:1), G. DPPC:CH (1:1), H. DPPC:CH:DCP (3:1:0.5)

[2] refer to Table 2-1 for [lipid composites].

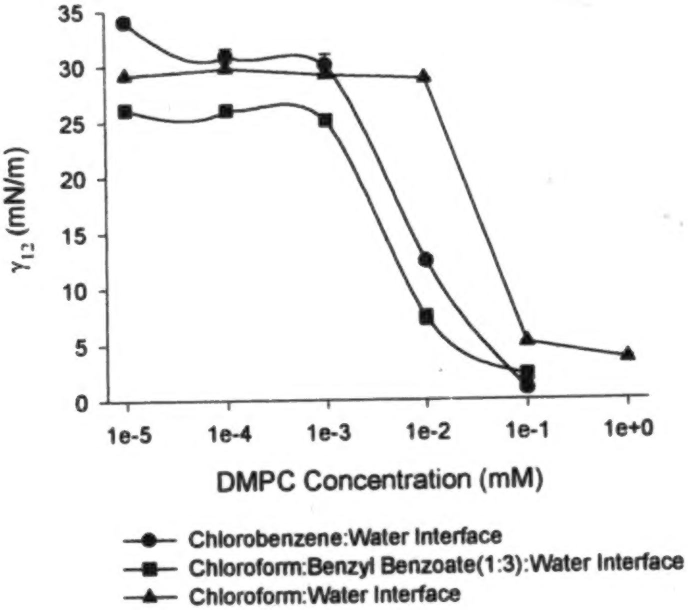

Figure 2-1. Interfacial tension vs. DMPC concentration in three solvent systems. Error bars represent s.d., n=6.

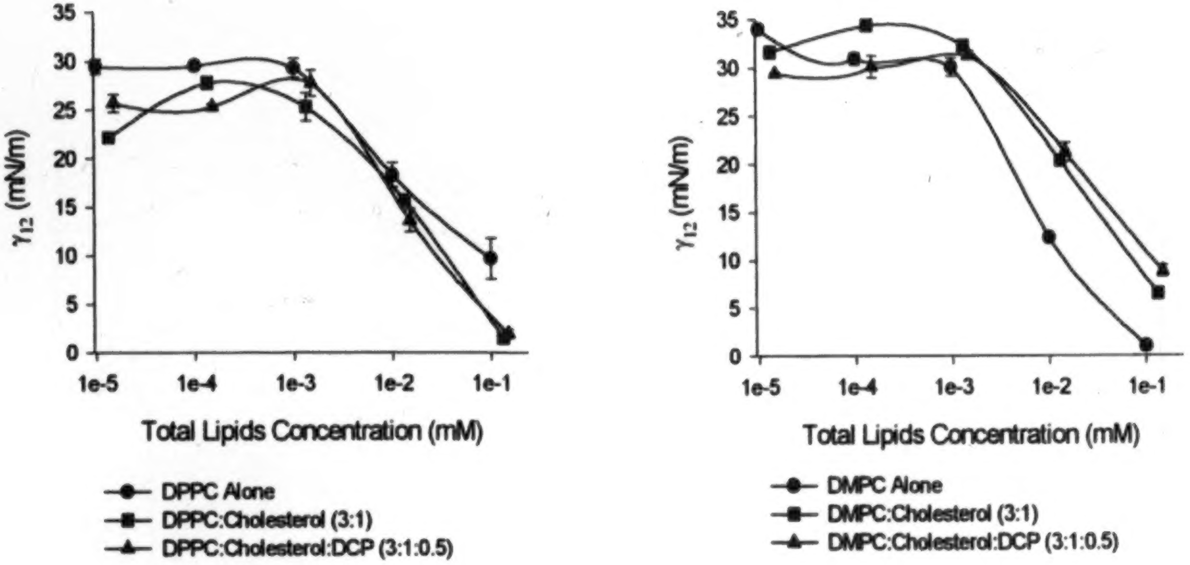

Figure 2-2. Interfacial tension ($\gamma_{12}$) effects due to lipid inclusion into a phosphatidycholine monolayer at the chlorobenzene/water interface. Error bars represent s.d., n=6.

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### *Chapter 3*

# FOLIC ACID-PEO-LABELED LIPOSOMES TO IMPROVE GI ABSORPTION OF ENCAPSULATED AGENTS

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## 3.1 Introduction

The design of targeted oral liposomes is anticipated to improve the systemic delivery of poorly absorbed agents, such as proteins and peptides. It is anticipated that exploitation of vitamin receptors present in the GI absorptive epithelia for transcellular delivery of liposome contents will improve the oral absorption of poorly-absorbed hydrophilic compounds through site-specific mechanisms. The following describes the design and characterization of a FA derivative for surface modification of liposomes.

## 3.2 Background

Reports based on folate-mediated cellular targeting, primarily for intracellular delivery, have been reviewed in Sec 1.5.2.3.2. The specificity and pH-dependence of folate absorption from the gastro-intestinal tract have been described (1). Caco-2 cells, representative of the GI tract, express folate receptors and are good models for folate-mediated drug delivery (2;3). Preliminary investigations using Caco-2 cell culture have determined that cytotoxic folate-conjugates are effectively endocytosed to elicit cellular responses (4), however the transcytotic flux of conjugates was not determined. Although oral liposome delivery via folate-mediated endocytosis has not yet been reported, there is substantial evidence that Caco-2 cell culture may be a good model for screening this potential application. In this regard, Lehr et al. (5) have recently advocated the use of the Caco-2 cell line as a potential tool for evaluating functionalized, active uptake of nanoparticles. This report demonstrates the utility of surface-adsorbed FA derivatives for improving the uptake and transport of a liposome-loaded non-permeable marker (TR-dex) across Caco-2 cell monolayers.

## 3.3 Materials and Methods

### *3.3.1 Synthesis of folic acid-poly(ethylene oxide) (FA-PEO) conjugates*

Poly(ethylene (bis-amine) oxide) (PEO, nominal MW 3350), pteroylglutamic (folic) acid (FA), dicyclohexylcarbodiimide, cholesterol chloroformate, and fluorescein isothiocyanate (FITC) were obtained from Sigma Chemical Co. (St.Louis, Mo). Reagent-grade DMSO, DMF and pyridine were distilled prior to use. The synthesis of folic acid-PEO conjugates (FA-PEO) was carried out at a 1:1 mole ratio (m.r.) under previously described conditions (6), dialyzed against 5 mM pH 8 borate buffer for 3 d (Spectra/Por® 3000 MW dialysis membrane, Spectrum, Houston, USA), and further purified by batch adsorption to a cellulose-phosphate resin in 5 mM pH 7.0 phosphate buffer to remove unreacted PEO. The suspension was filtered to remove the resin and the clear filtrate was lyophilized for 12 h. The lyophilized powder was identified by NMR, IR, and mass spectrometry. FA-PEO and PEO were tagged with FITC in a pH 8.0 borate buffer followed by dialysis (Spectra/Por® 3000 MW dialysis membrane) for 24 h until no appearance of FITC in the dialysate was detected. Confirmation of the removal of unreacted FITC from the product was the disappearance of the S=C=N band in the IR spectra.

CH was conjugated with FA-PEO (FA-PEO-CH) by reacting a 10-fold molar excess of cholesterol chloroformate with FA-PEO solubilized in dry DMF and dry DMSO, respectively, in the presence of dry pyridine for 24h at room temperature in the dark. The solution was dialyzed against pure DMSO (Spectra/Por® 3000 MW dialysis membrane) for 24h. The dialyzed solution was filtered (0.22 μm) to remove unreacted CH and the filtrate was lyophilized. The identity of the product was confirmed by NMR.

### *3.3.2 Preparation of TR-dextran (3000 MW) liposomes*

Distearoylphosphatidylcholine (DSPC) and dimyristoylphosphatidylglycerol (DMPG, Na salt) were obtained from Princeton Lipids (Princeton, NJ), CH from Sigma Chemical Co. (St.Louis, Mo), Texas Red® dextran 3000 MW (TR-dex) from Molecular Probes, Inc. (Eugene, OR), and cetyltrimethylammonium bromide (CTAB) from Aldrich Chemical Co., Inc. (Milwaukee, WI). DSPC:CH:DMPG (3:1:0.25 m.r.) was dissolved in 1:9 (v/v) methanol:chloroform and rotary-evaporated to form a thin lipid film on the walls of a round-bottom flask. The film was hydrated with double-distilled water and the suspension was probe-sonicated (W-375 Ultrasonicator, Heatsystems-Ultrasonics, Plainview, NJ) at 55°C for 5 min resulting in a translucent aqueous dispersion of small unilamellar vesicles (SUVs, ~35 mM lipids). The lipid dispersion was flash-frozen and lyophilized overnight prior to use. DRVs were prepared by adding 500μL of a concentrated solution of TR-dex (~0.5 mg/mL) in Hepes buffered saline (HBS, pH 5.8) to the dehydrated lipids and vortexing. The gel was then diluted with 4.5 mL HBS to a final lipid concentration of ~35 mM. The DRVs were serially-sized by individual passes through 1.0 μm, 0.4 μm, and 0.2 μm polycarbonate membrane filters in a Lipex® Extruder (Lipex Biomembranes Inc., Vancouver, Canada) at 65°C. Size analysis of the resulting liposomes was performed using a BI-90 laser light scattering particle sizer (Brookhaven Instruments Corp., Holtsville, NY). Finally, gel permeation chromatography (GPC, Sephadex G-50, 1.5 x 15cm) was used to separate untrapped TR-dex from liposomes.

Preparation of FA-PEO-CH-coated liposomes (DSPC:CH:DMPG:FA-PEO-CH, 3:1:0.25:0.05 m.r.) was also carried out according to the above procedure. FA-PEO-CH was incorporated with the lipids prior to lyophilization.

### 3.3.3 Encapsulation Efficiency Determination

Liposomes were solubilized in 20 mM CTAB solution at 60°C prior to analysis. Encapsulation efficiency (EE) of VCM in liposomes was determined from:

$$\%EE = \frac{C}{C_o} \times 100, \tag{1}$$

where C is the concentration of TR-dex in the liposome dispersion (accounting for GPC dilution if necessary), and $C_o$ is the original concentration of TR-dex added.

### 3.3.4 Release Studies

Release of TR-dex from the liposomes was assessed by dialyzing an aliquot of TR-dex DRVs (Spectra/Por®, 10 000 MW dialysis membrane) in 100 mL fresh pH 5.8 HBS at 37°C under moderate magnetic stirring conditions then measuring the dialysate for the presence of TR-dex over 2 h.

### *3.3.5 Caco-2 cell culture*

Caco-2 type $BBE_1$ cells (a gift from Dr. M. Mooseker, Yale University) were grown in folate-free Dulbecco's Modified Eagles Medium, supplemented with 10% fetal bovine serum, 2 mM glutamine, and 0.002% insulin. Cells were seeded at $2 \times 10^6$ cells between passages 61-78 on high-density, collagen-coated 0.45 μm (PEO-conjugate transport) or 1.0 μm (liposome transport) pore-size Falcon PET inserts (24 mm, Becton Dickinson, Oxford, UK) and used between days 18-21 for transport studies. Tight junction formation was monitored by immunofluorescent labeling of the tight junction protein ZO-1. Integrity of cell monolayers was confirmed by transepithelial electrical resistance (TER) (Millicell-ERS, Millipore Co., Bedford, USA) and the transepithelial flux of a non-transported 3000 MW TR-dex probe. Transport studies were conducted in Hepes-buffered saline with 1 g/L glucose, 1.3 mM $CaCl_2$, and 0.5 mM $MgCl_2$ (HBSG+) at pH 5.8 for a 2 or 4 h-period. Apparent permeability ($P_{app}$) of the cells to PEO-FITC, FA-PEO-FITC, TR-dex, or liposomal TR-dex was calculated as follows:

$$P_{app} = \left( V / A \bullet C_o \right) \times \frac{dC}{dt} \text{ (cm/s)}, \qquad (2)$$

where V • (dC/dt) is the steady-state rate of appearance of apically-applied conjugates in the basal medium, $C_o$ is the initial conjugate concentration in the apical medium, and A is the surface area of the membrane. Steady-state flux (sink condition) was maintained throughout the course of the studies due to a high concentration gradient across the monolayer and a continual basal chamber diffusate concentration less than 10 percent of saturable concentration.

### *3.3.6 Analytical*

Fluorescent analysis was conducted in a DMF-3000 spectrofluorometer (Spex FluoroMax, USA). FITC conjugates were analyzed by measuring the monochromatic fluorescence at 490ex / 518em λ, and TR-dex was measured at 592ex / 609em λ, according to the maximum excitation (ex) / emission (em) spectra.

Fluorescent microscopy of the Caco-2 monolayers was performed with a microscope (Zeiss Axioskop, W. Germany) fitted with epifluorescence and FITC and TR-dex filters. Following each transport experiment, the cell monolayers were washed twice with fresh transport media to remove adsorbed liposomes, three times with pH 7.4 phosphate buffered saline (PBS, 4°C), fixed with a 2.5 % paraformaldehyde solution (4°C, 30 min), and then washed three times with PBS (4°C). A thin piece of the cell filter was cut and mounted in anti-bleaching media on a slide for microscopic examination of intracellular TR-dex.

To visualize tight junctions, Caco-2 cells were fixed to inserts and permeabilized with methanol (-20°C) to expose ZO-1. Immunofluorescent labeling of ZO-1 was carried out with an initial incubation period with polyclonal anti-human ZO-1 (Zymed, USA) followed by secondary fluorescent labeling with anti-rat-FITC (Jackson Immunoresearch Lab., USA). A thin piece of cell filter was cut and mounted as described in the previous section for fluorescent microscopy.

Infrared (IR) analysis (Nicolet Magna 55 Series II) was performed with KBr disks of the FA-PEO conjugates.

The NMR spectra of the PEO-conjugates dissolved in DMSO-$d_6$ were recorded on a Bruker AM 300 FT spectrometer (Oxford/Spectrospin Industries, Canada). Chemical shifts were reported in ppm ($\delta$, TMS).

Mass determination of PEO-conjugates was determined using quadrapole electrospray mass spectrometry (Fisons VG Trio 2000, Cheshire, England) with a cone voltage of 40 V.

### 3.4 Results and Discussion

Figures 3-1, 3-2, and 3-3 illustrate the successful derivatization of PEO with FA from evidence of IR, mass spectrometry, and NMR. The IR data (Fig. 3-1 I, II) illustrate FA derivatization by the appearance of a large absorption peak (3000-3600 $cm^{-1}$) corresponding to the glutamic acid residue of FA. The PEO precursor had a nominal MW of 3329 (reported as 3350 MW, Sigma Chemical Co.) with a wide size distribution as shown by the mass spectrometry data. Thus, precise mass determination of the derivatives cannot be determined although an increased trend in MW was identified (Fig. 3-2 I, II). Figures 3-3 I, II, and III represent the NMR spectra of FA-PEO, PEO, and FA-PEO-CH, respectively. The large proton peak at 3.3 ppm in the NMR spectrum (Fig. 3-3 II) is due to the ethylene protons of the PEO backbone, and the downfield proton peaks produced by the conjugate are representative of FA (4-10 ppm, Fig. 3-3 I). Figure 3-3 III provides NMR evidence of the formation of FA-PEO-CH where the upfield proton peaks of cholesterol appear (0.5-2.4 ppm, cf. Fig. 3-3 I).

Encapsulation efficiency of TR-dex in uncoated (DSPC:CH:DMPG, 3:1:0.25 m.r.) and FA-PEO-CH-coated (DSPC:CH:DMPG:FA-PEO-CH, 3:1:0.25:0.05 m.r.) DRVs ranged from 20-25 percent after extrusion, uninfluenced by the coating. The release of TR-dex from the DRVs at 37°C resulted in < 5 percent of the dose being released over 2 h. Thus, the low incidence of free TR-dex was not a confounding variable when interpreting liposomal TR-dex transepithelial flux.

Confluence of the cell monolayers was confirmed by mean pre-experimental TER of 480 ± 99 $\Omega\bullet cm^2$ and an average TR-dex $P_{app}$ of 1.64 ± 0.50 x $10^{-7}$ cm/s. Figure 3-4 illustrates staining of the ZO-1 tight junction protein, confirming formation of tight junctions between cells. Transport studies were initially conducted at pH 5.8, corresponding to the optimal pH for absorption of folates from the GI tract. Table 3-1 shows an 8-fold improvement in the $P_{app}$ of the FA-PEO conjugate compared to underivatized PEO, resulting in 16 percent of the FA-PEO dose being transported through the cell monolayer (basal recovery). The $P_{app}$ of underivatized PEO was similar to that of TR-dex, indicating no specific uptake mechanism involving PEO (2.84 vs. 1.64 x $10^{-7}$ cm/s, respectively). Total recovery of the FITC-label was obtained, thereby discounting the possibility of "bio-fouling" of the fluorescent measurement. Total fluorescence was found to be approximately only 15% in excess in both cases, which does not account for the 8-fold difference in $P_{app}$ observed. Figure 3-5 compares the relative $P_{app}$ of PEO and FA-PEO over a 4 h period. Table 3-2 provides evidence that the FA-PEO conjugate appears to retain specificity for the FA-binding protein at the apical cell surface. Transport of the FA-PEO conjugate was inhibited at a higher pH (HBSG+, pH 7.4) and by the presence of excess free FA (35 μM).

The development of liposome vectors for oral delivery is aimed at improving the bioavailability of poorly-absorbed agents, such as proteins and peptides. Such liposome systems are advantageous in providing depots of high drug loads in comparison to that obtained by chemical conjugation. TR-dex, a poorly-absorbed, neutral, hydrophilic, large MW marker enabled elucidation of a liposome system across Caco-2 monolayers. The nominal size of the liposomes was 250 nm (polydispersity of 0.244 and 0.199 for uncoated and coated liposomes, respectively), which has been considered optimal for colloidal particle uptake by GI eptithelia (7;8). Figure 3-6 illustrates the $P_{app}$ of free and encapsulated TR-dex in uncoated and FA-PEO-CH-coated liposomes ($TR_{free}$, $TR_{uncoat}$, $TR_{coat}$, respectively). The results in Table 3-3 indicate that simple encapsulation of TR-dex by DSPC:CH:DMPG (3:1:0.25 m.r.) liposomes did not significantly improve the transport of TR-dex across Caco-2 monolayers. However, a significant 5-fold increase in flux of TR-dex was observed from 0-30 min post-administration for $TR_{coat}$ compared to $TR_{uncoat}$ and $TR_{free}$. There were no significant differences in the apparent flux at later time points, possibly due to saturation of the folate-binding receptors. The rapid initial flux of $TR_{coat}$ at early times is indicative of receptor-mediated routing (9). Unfortunately, the full potential of FA-coated liposomes could not be explored by this in vitro technique because the 1μm pore-size PET filters were a barrier to liposome transport, likely contributing to an artificially low liposomal-TR-dex $P_{app}$. Transport of TR-dex alone using collagen-coated PET inserts, indicated that the flux of TR-dex in liposomes was inhibited by the liposome carrier (data not shown). Interestingly, the presence of cells improved the transport of liposomal-TR-dex across PET inserts, suggesting that when coated-liposomes are used the improvement in uptake of TR-dex is due to intracellular processing and release of TR-dex that subsequently passes through the basal side of the cell monolayer. In comparison, a previous report using an isolated, perfused, intestinal-loop model has indicated that liposomes composed of higher $T_m$ lipids are internalized to a greater extent than liposomes consisting of lower $T_m$ lipids, although once intracellular absorption has occurred liposomes of high $T_m$ lipids are more extensively metabolized (10). In order to further characterize liposome uptake, cells were fixed to PET inserts after transport studies, cell surfaces were washed of adsorbed (non-internalized) liposomes, and the cells were examined by fluorescence microscopy to detect intracellular TR-dex. Figures 3-7A and B illustrate intracellular TR-dex following $TR_{uncoat}$ and $TR_{coat}$ administration, respectively. The large increase in intensity of fluorescence derived from the $TR_{coat}$ system was considered to be evidence of improved TR-dex uptake within the FA-coated liposomes. Comparison of intracellular TR-dex accumulation with the $P_{app}$ data suggests uncoupling of cellular uptake and transepithelial transport, indicating only a fraction of the absorbed dose may be available for transcytosis.

### 3.5 Conclusions

There are now indicators that FA-PEO-labeled liposomes have the potential for improving the GI transport of agents contained therein. In the present context, the FA-PEO conjugate retained its biological specificity for the FA-binding protein of the membrane in agreement with previous reports (11;12). In addition, cellular recognition of the FA component was retained after incorporation at liposomal surfaces with the result that, after binding, the liposome contents were internalized by receptor-mediated endocytosis based on evidence of enhanced transcellular transport at early times, and inhibition of FA-PEO-FITC transport by free FA and an increase in pH. Consequently, an increase in flux of a poorly-absorbed, neutral, hydrophilic, large MW marker (TR-dex) across cultured Caco-2 cell monolayers was observed. On this basis, transport should be a function of GI pH and the degree of cellular FA-binding protein expression. However, the results of in vitro studies should be interpreted cautiously because Caco-2 cell culture is a transformed cell-line with oncogenic lineage and therefore may have increased FA-binding protein expression, compared to normal GI epithelia due to the increased requirement of these cells for nucleotide synthesis. On the other hand, the observed enhancement of TR-dex flux conferred by FA-coated liposomal delivery may be artificially low due to the low permeability imparted by the PET filters to liposome transport. Furthermore, a lower permeability (increased TER) for Caco-2 monolayers compared to the GI tract may provide an expectation that transport in vivo could be even greater. Future work should attempt to delineate mechanisms of liposome-associated drug absorption and subsequent transcytosis and to correlate in vitro observations to in vivo performance.

Table 3-1. Apparent permeability ($P_{app}$) of Caco-2 monolayers to PEO-FITC and FA-PEO-FITC conjugates.

| | $P_{app}$[a] (x $10^7$cm/s) | Basal Recovery[a] (%) | n |
|---|---|---|---|
| FA-PEO-FITC | 23.79 ± 5.99 | 16.23 ± 3.99 | 6 |
| PEO-FITC | 2.84 ± 0.45 | 1.92 ± 0.31 | 4 |

[a] mean ± s.d. in a 4 h study.

Table 3-2. Selective inhibition of apparent permeability ($P_{app}$) of Caco-2 cells to FA-PEO-FITC in various media[a].

| | $P_{app}$[b] (x $10^7$ cm/s) | Basal Recovery[b] (%) | n |
|---|---|---|---|
| HBSG +, pH 7.4 | 2.87 ± 3.28 | 1.40 ± 1.23 | 4 |
| HBSG+, pH 5.8, free FA (35μM) | 5.11 ± 0.50 | 1.73 ± 0.17 | 4 |

[a] c.f. values in Table 3-1 for FA-PEO-FITC control at pH 5.8.

[b] means ± s.d. in a 2 h study.

Table 3-3. Apparent permeability ($P_{app}$) of Caco-2 cells to free and liposome-encapsulated TR-dex.

| Formulation | $P_{app}$[a] |
|---|---|
| $TR_{free}$ | 1.57 ± 0.19 |
| $TR_{uncoat}$ | 1.11 ± 0.57 |
| $TR_{coat}$[b] | 5.73 ± 1.07 |

[a] mean ± s.d., n=3 in HBSG+ pH 5.8 over a 30 min interval.

[b] significant difference (one-way ANOVA, Tukey post-hoc comparison, $p < 0.05$)

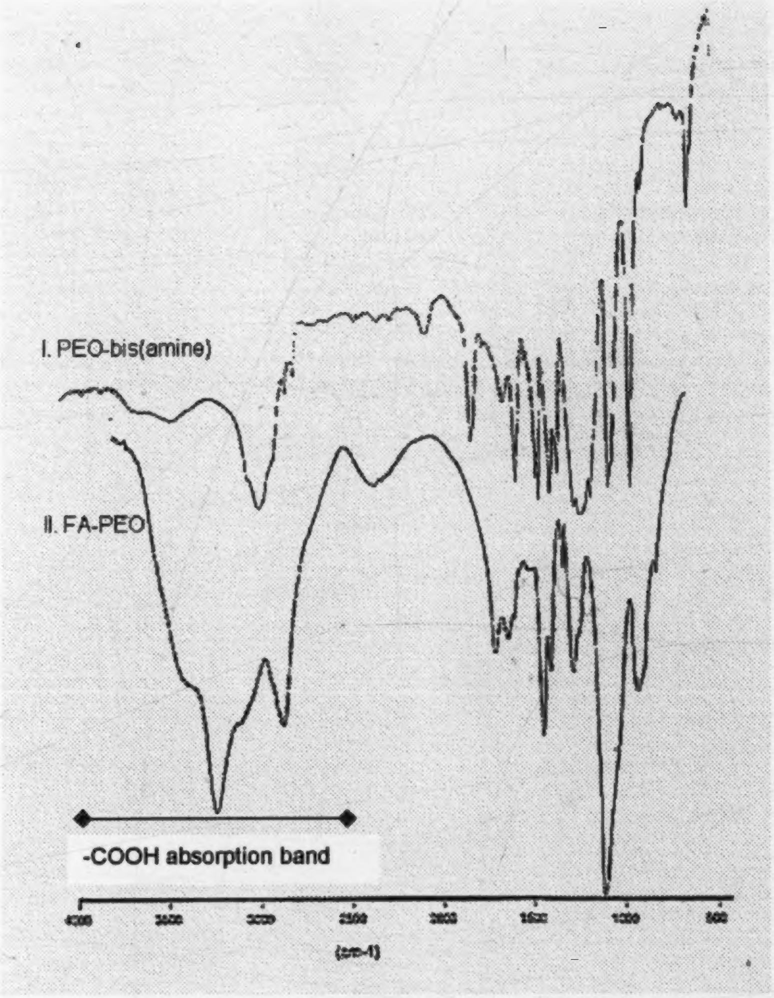


Figure 3-1. IR analysis of KBr disks of (I) PEO and (II) FA-PEO demonstrating the large –COOH absorption band (c.f. 3000-3600 $nm^{-1}$) due to the glutamic acid residue of FA following derivatization.

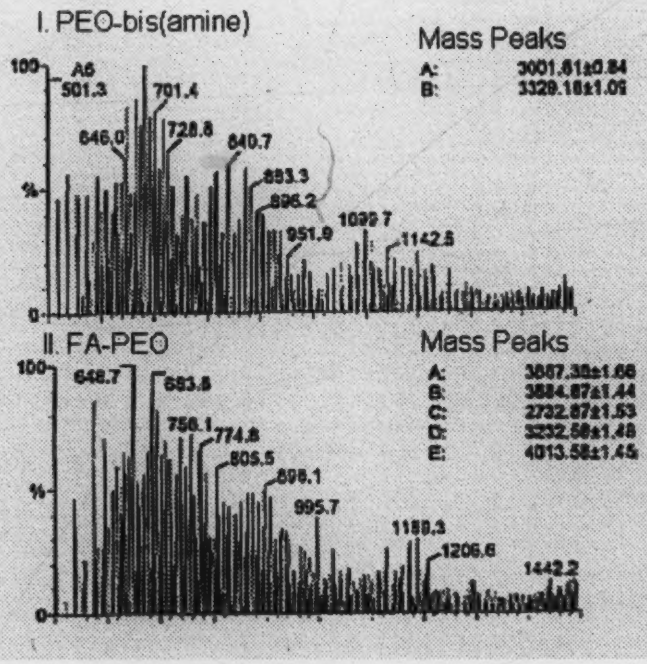


Figure 3-2. Electrospray Mass spectrum analysis of (I) PEO and (II) FA-PEO indicating an increase in average mass of ~400 daltons for the FA-PEO derivative. (PEO-bis(amine) had an initial wide MW distribution as supplied from the distributor).

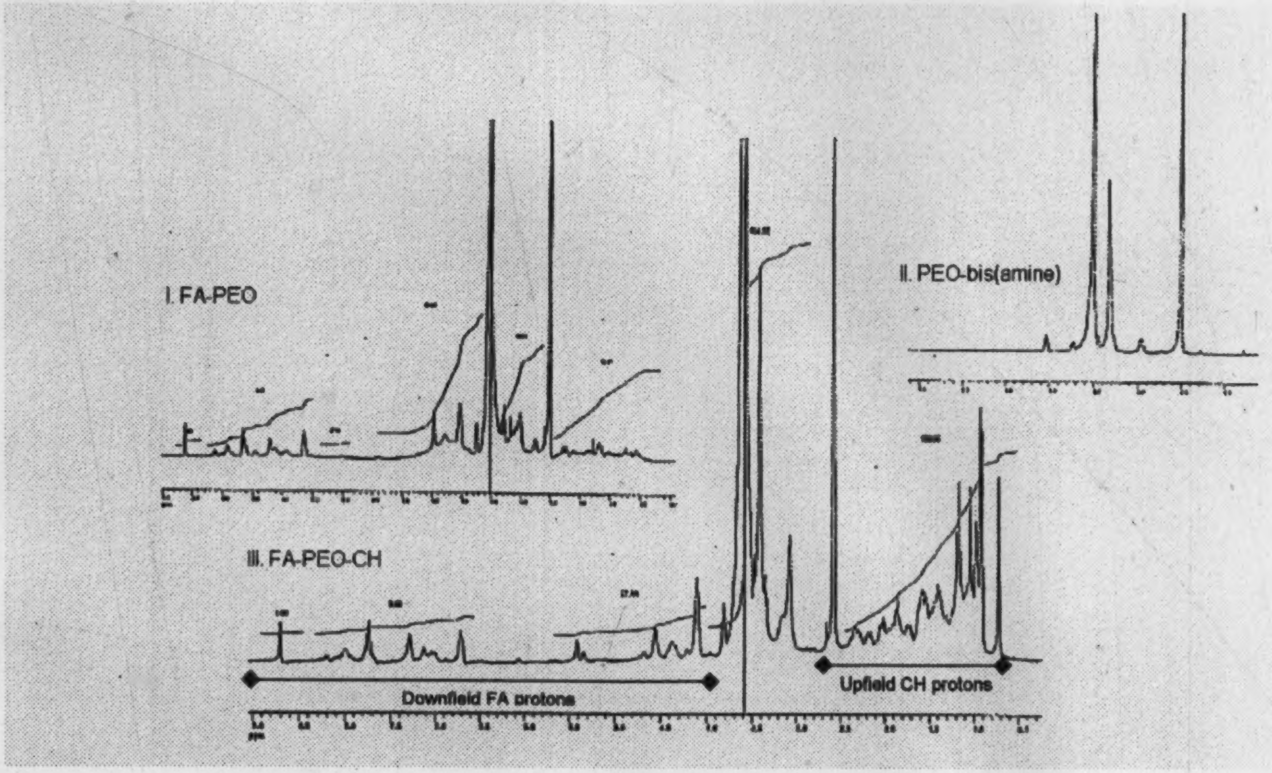
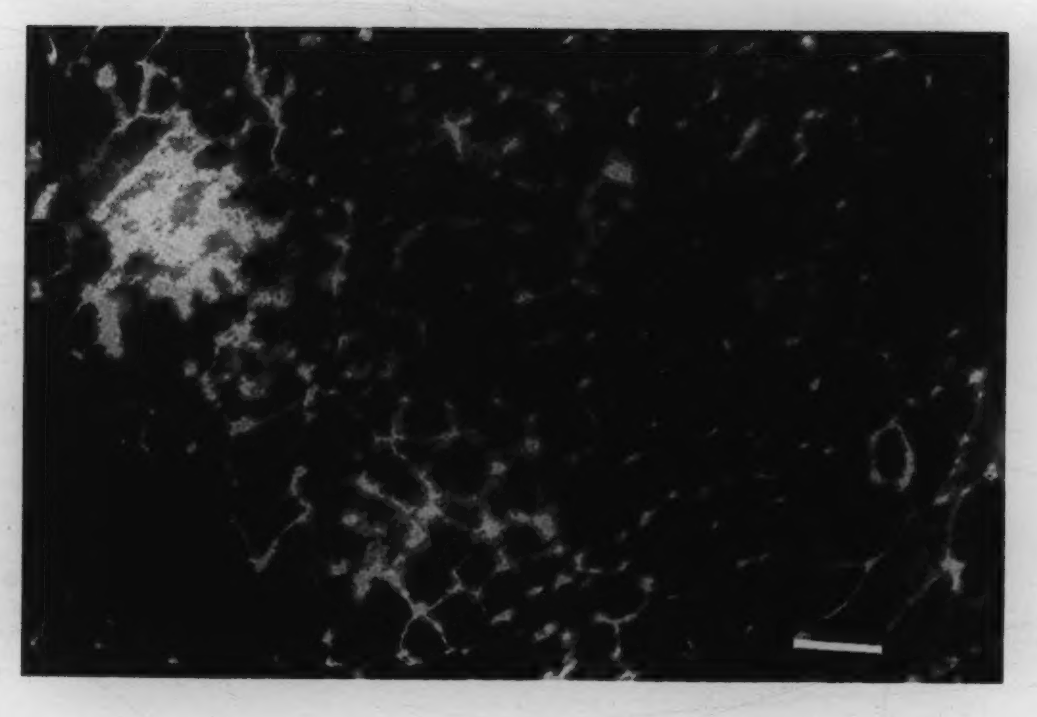


Figure 3-3. H$^1$-NMR analysis of (I) FA-PEO, (II) PEO, and (III) FA-PEO-CH indicating FA and CH derivatization to PEO.

Chemical shifts reported in ppm (δ, TMS). All samples dissolved in DMSO-$d_6$.

Figure 3-4. Immunofluorescent micrograph of FITC-labeled ZO-1 tight junction protein of Caco-2 cell monolayer. Fluorescence at tight junction represented by light green color. Formation of tight junctions is evidence of cellular contacts indicating monolayer confluence and integrity. Magnification 160 X. (bar = 50μm).



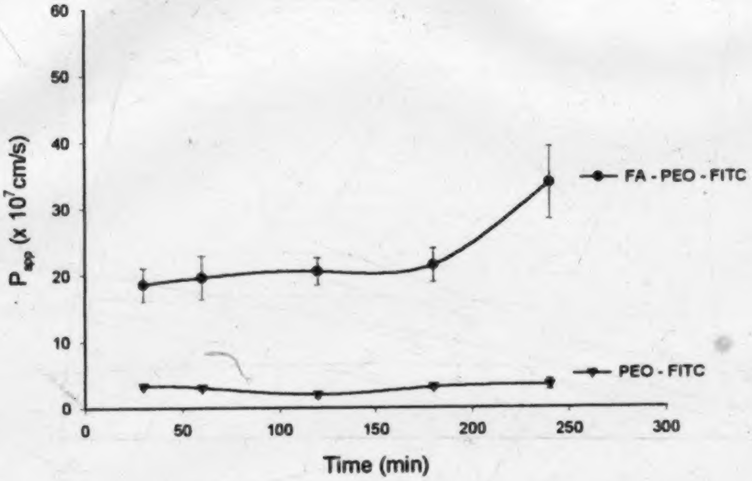


Figure 3-5. Apparent permeability of FA-PEO-FITC (±SEM, n=6) and PEO-FITC (±SEM, n=4) across Caco-2 monolayers over 4 h in HBSG+, pH 5.8 at 37°C.

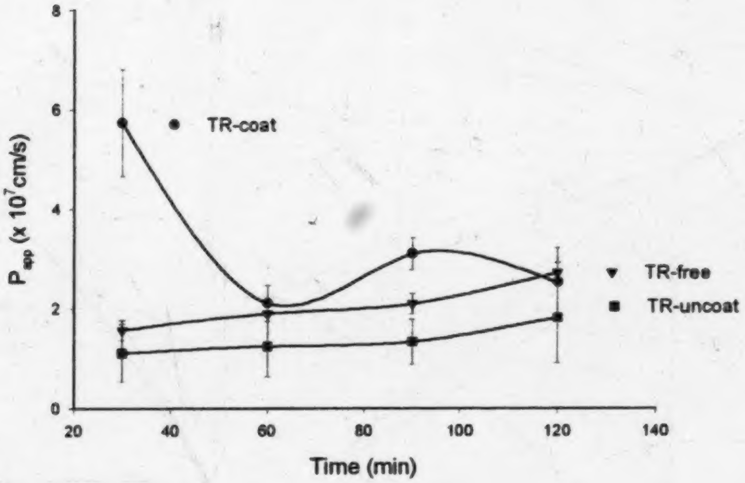
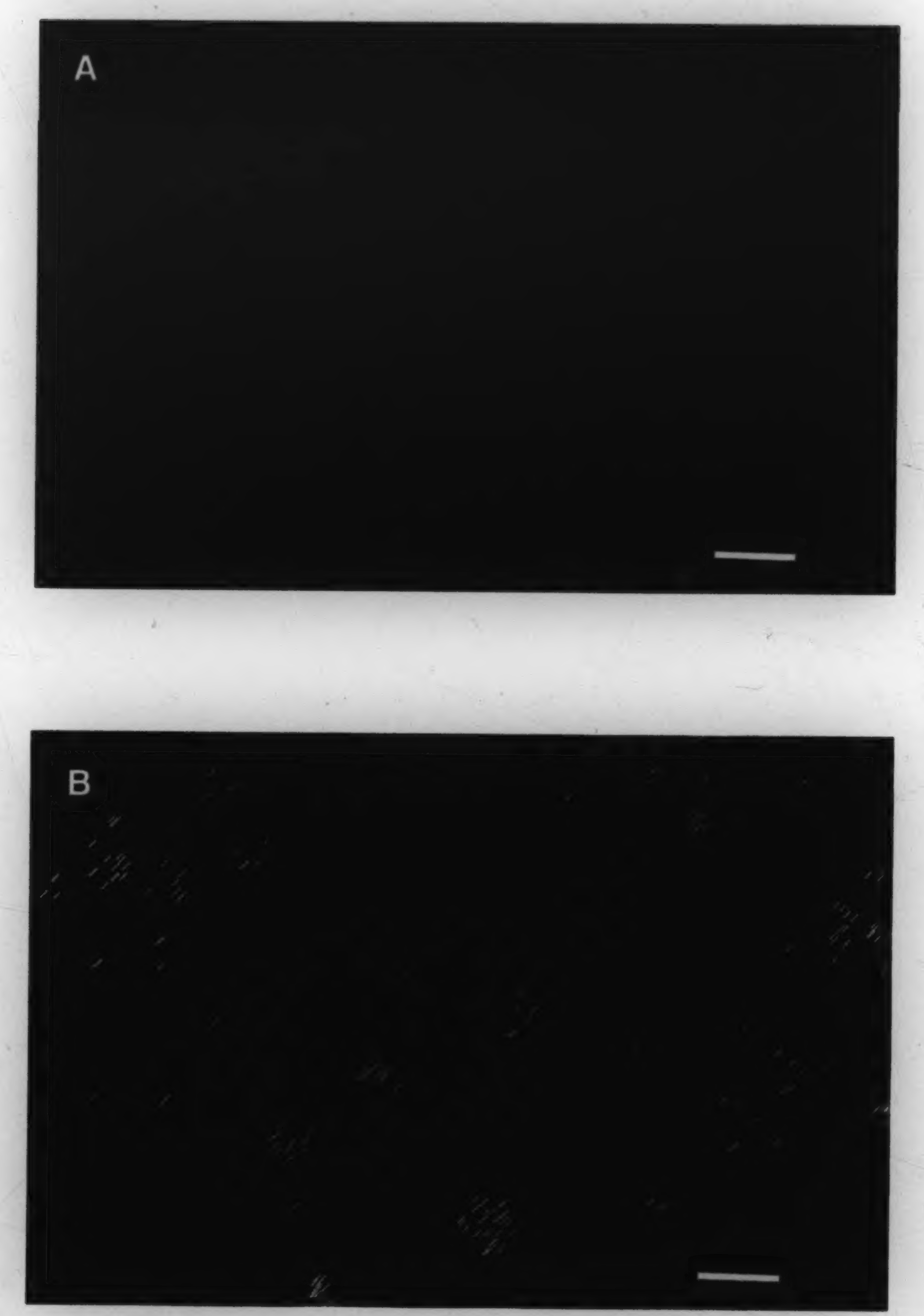


Figure 3-6. Apparent permeability of FPC-coated liposomal TR-dex (±SEM, ●, n=7), uncoated liposomal TR-dex (±SEM, ■, n=4), and free TR-dex (±SEM, ▼, n=4) across Caco-2 monolayers over 2 h in HBSG+, pH 5.8 at 37°C.

Figure 3-7. Fluorescent micrograph of intracellular TR-dex delivered by (A) uncoated TR-dex liposomes, and (B) FA-PEO-CH-coated TR-dex liposomes. TR-dex fluorescence illustrated by red color. Fluorescence was below the plane of the tight junctions (c.f. Fig. 3-4). Cell surfaces were washed of adsorbed (unabsorbed) liposomal TR-dex prior to fixation and analysis. Magnification 160 X. (bar = 50μm).


A
B

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## *Chapter 4*

# FORMULATION AND IN VITRO EVALUATION OF TARGETED ORAL VANCOMYCIN LIPOSOMAL DOSAGE FORMS

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## 4.1 Introduction

Successful delivery of oral protein/peptide liposome systems depends on formulation strategies designed to achieve good intestinal stability (of liposome and drug), greater GI wall affinity, and ample protein/peptide loading at the site of delivery. The following describes the optimization of VCM loading into liposome carriers, and characterization of developed systems for selection of dosage forms for pre-clinical testing. In particular, FA-targeted liposomes have been investigated.

## 4.2 Background

VCM, a glycopeptide antibiotic, was chosen as a model for peptide absorption due to its physico-chemical profile including a molecular weight greater than the cut-off for paracellular transport (1485.7), multiple charge sites (pI 7.0), and high hydrophilicity ($S_0 \approx 15$ mg mL$^{-1}$ at pI, RT). VCM solubility ranges from 15 – 100 mg mL$^{-1}$ depending on the pH of the environment. VCM is poorly-absorbed from the GI tract (bioavailability < 2 percent) and is predominately cleared by the kidneys (> 95 percent), making it an excellent model compound for assessing the effect of liposomes on the GI transport of proteins/peptides without the confounding variable of first-pass metabolism (e.g. insulin) (1-3). The results of this study are intended to provide insight of the potential of liposomes to improve the GI flux of VCM as a model peptide using an in vitro intestinal cell model.

## 4.3 Materials and Methods

### *4.3.1 Materials*

Distearoylphosphatidylcholine (DSPC) and dimyristoylphophatidylglycerol (DMPG, Na salt) were obtained from Princeton Lipids (Princeton, NJ). Cholesterol (CH), dicetylphosphate (DCP), sodium cholate (NaC), sodium chenodeoxycholate (NaCDC), sodium deoxycholate (NaDC), and vancomycin (VCM) were obtained from Sigma Chemical Co. (St. Louis, Mo), and cetyltrimethylammonium bromide (CTAB) from Aldrich Chemical Co., Inc. (Milwaukee, WI). FA-PEO-CH was synthesized according to Chap. 3.

### *4.3.2 Liposome Preparation*

DSPC, CH, DMPG, and DCP were dissolved in 1:9 (v/v) methanol:chloroform and rotary-evaporated to form a thin lipid film on the walls of a round-bottom flask. Mole ratios (m.r.) of the negatively-charged lipids (DMPG, DCP) were varied, while the DSPC:CH m.r. was held constant at 3:1. Multilamellar vesicles (MLVs) were prepared by film hydration with an aqueous solution of VCM at either pH 4.0 (Walpole acetate buffer) or 7.0 (Sørenson's phosphate buffer, SPB) to a final lipid concentration of 35 mM. DRVs were prepared by hydrating the lipid film with double-distilled water and sonicating the 35 mM lipid suspension (W-375 ultrasonicator, Heatsystems-Ultrasonics, Plainview, NJ) at 55°C for 5 min resulting in a translucent aqueous dispersion of SUVs. The lipid dispersion was flash-frozen and lyophilized overnight prior to use. DRVs were re-constituted by adding 500μL of a concentrated solution of

VCM in SPB (pH 7.0) to the dehydrated lipids and vortexing. The gel was then diluted with 4.5 mL SPB to a final lipid concentration of 35 mM. The DRVs were serially-sized by individual passes through 1.0 µm, 0.4 µm, and 0.2 µm polycarbonate membrane (Lipex® Extruder, Lipex Biomembranes Inc., Vancouver, Canada) at 65°C. Size analysis of the resulting liposomes was performed using a BI-90 laser light scattering particle sizer (Brookhaven Instruments Corp., Holtsville, NY). Finally, gel permeation chromatography (GPC, Sephadex G-50, 1.5 x 15cm) was used to separate free and liposome encapsulated VCM.

DRVs of DSPC:CH:DCP prepared as described were vortex-mixed with FA-PEO-CH, yielding FA-coated liposomes of DSPC:CH:DCP:FA-PEO-CH (3:1:0.25:0.05, m.r.).

### *4.3.3 Encapsulation Efficiency*

Refer to Section 3.3.3.

### *4.3.4 Drug Release*

Release of VCM from liposomes was assessed by dialyzing a 0.5 – 1.0 mL aliquot of VCM liposomes (Spectra/Por®, 10 000 m.w. dialysis membrane) against 50 mL freshly-prepared simulated gastric fluid, USP (SGF, pH 1.2), simulated intestinal fluid, USP (SIF, pH 7.4), SIF with 10 mM sodium cholate (SIF+), or SIF with a 10 mM bile salt cocktail of NaC, NaCDC, and NaDC (2:2:1, m.r) (SIF+BSC) at 37°C under moderate magnetic stirring conditions then measuring the dialysate for the presence of VCM over a period of 2 h. Average bile acid concentrations of 10 mM are considered to be similar to human intestinal bile concentrations, although bile acids are generally conjugated to glycine or taurine in vivo (4).

### *4.3.5 Differential Scanning Calorimetry (DSC)*

Liposomes were analyzed by DSC (SSC/5200 SII DSC 120, Seiko Instruments Corp., USA) to assess the effect of lipid composition, and VCM or FA-PEO-CH inclusion on the $T_m$ of native DSPC liposomes. Thermograms were obtained by heating samples at 2°C/min from 25-100°C. Thermal data analysis was carried out with a HP 9000 series 700 workstation (Hewlett Packard, Palo Alto, CA) equipped with HP VUE® and a DSC module.

### *4.3.6 Caco-2 Cell Culture*

Caco-2 type $BBE_1$ cells were cultured as described in Section 3.3.5. Cells were seeded at $2 \times 10^6$ cells between passages 79-81 on high-density, collagen-coated, 1.0 μm pore-size Falcon PET inserts (24 mm, Becton Dickinson, Oxford, UK) and used between days 7-10 for transport studies[1]. Cells attached to filters were fed daily with 10 percent FBS supplemented FFDMEM for days 6-9 and fed FA-depleted media for 1d prior to transport studies. FA-depleted media consisted of FFDMEM supplemented with 10 percent FBS which was pre-treated with activated charcoal to remove endogenous folates (5). The $P_{app}$ of VCM across Caco-2 cell culture was calculated according to Section 3.3.5.

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[1] Reduced culture duration from Chap. 3 justified according to equivalent FA-PEO-FITC $P_{app}$.

### *4.3.7 Analytical*

High-performance liquid chromatography coupled with UV detection (Lambda-max model 481, Waters, Milford, MA) at 229 nm was used to quantitate VCM samples. The mobile phase was prepared by premixing SPB (pH 7.0):acetonitrile (95:5, v/v), degassed, and pumped (Model 501 HPLC pump, Waters) through a cyano (–CN) column (Radial Pak cartridge, 8 x 10 RCM, Waters) at a flow-rate of 1mL/min. All reagents were HPLC grade. VCM samples were diluted as necessary and 100 μL samples were injected on to the column directly via an auto-sampler (712 Wisp, Waters). Samples containing liposome dispersions were pretreated in 20 mM CTAB at 60°C to solubilize liposomes prior to VCM analysis. The concentration of VCM was calculated from a calibration graph (peak area vs. concentration).

## 4.4 Results and Discussion

The primary objectives involved in formulation of oral liposome systems are GI stability, GI affinity, and drug loading. Numerous techniques to improve the stability of liposomes have been advanced, and are reviewed in Section 1.5.1. DSPC was chosen as an inherently stable ($T_m$ = 55.1°C) phospholipid platform on which to base oral VCM liposome formulations. A choice of high $T_m$ lipids minimizes components necessary to stabilize the vesicles, particularly to bile salts. CH addition to the liposome bilayer increases liposome stability, decreases fluidity of the hydrocarbon chains, and reduces membrane permeability, effectively plasticizing the membrane (6). A negatively-charged lipid facilitates liposome formation due to electrostatic repulsion and has been implicated in improving GI affinity (7). Furthermore, a negatively-charged bilayer component can improve the loading of cationic molecules by ionic interaction, which would occur for VCM loading at a pH $< 7.0$.

Table 4-1 reports EE of VCM in liposomes with two negatively-charged lipids (DMPG, DCP) at pH 4.0 or 7.0 prepared using the traditional film hydration (MLVs) or the dehydration-rehydration process (DRVs). MLV preparation resulted in poor overall EE of VCM regardless of lipid composition or pH of the VCM loading solution. DMPG-containing MLVs were unaffected by pH whereas DCP-containing MLVs trapped more VCM at the lower pH (3-fold improvement). Addition of a negative-charge to the bilayer did not substantially improve the loading of VCM in MLVs compared to neutral MLVs. VCM is a large m.w. hydrophilic glycopeptide and therefore the extent of liposome encapsulation of VCM depends on both the volume of the internal aqueous compartments of the liposomes and molecular accommodation between bilayers. This point is somewhat clarified by the DSC thermograms (Fig. 4-1) which demonstrates that VCM loading did not affect the $T_m$ of the DSPC liposome composition, from which it may be interpreted that VCM does not interact significantly with the lipid bilayer. In contrast, the addition of 25 mole percent CH abolished the $T_m$.

To improve the loading of VCM the DRV technique was employed, which has proven to dramatically enhance the loading of compounds of similar physical properties (8). Encapsulating VCM in DRVs resulted in a 25-fold increase in EE (when DCP was included) and was optimal at pH 7.0. The unexpected reduction of EE at pH 4.0 likely resulted from premature aggregation of VCM with DMPG or DCP in the highly-concentrated lipid gel prior to liposome formation.

To further optimize the liposome formulation for oral use, the size-range of particles was reduced by extrusion through 200 nm filters, which is a size consistent with reports of optimal particulate uptake from the GI tract (9). Table 4-2 lists the EE of VCM after liposome extrusion and removal of free VCM by GPC in various DRV formulations. A 67 percent reduction in EE of VCM occurred following extrusion, which is likely due to the reduction in available internal aqueous space of the smaller vesicles. Table 4-3 describes the effect of drug concentration on the EE of VCM in two negatively-charged DRVs. It can be seen that EE was not substantially affected by concentration, contributing to the notion that VCM entrapment is independent of lipid:drug ratios, and is affected predominantly by the volume of the aqueous compartments. VCM did not significantly alter the osmolarity of the loading solution at all concentrations tested.

The results of in vitro release of VCM from liposomes after a 2-h incubation period in simulated GI fluids at 37°C are presented in Table 4-4. The liposome formulations were relatively stable to pH change and to the addition of bile salts, including a 10 mM bile salt cocktail of NaC, NaCDC, and NaDC, where a maximum of only 40 percent of the drug was released. Liposomes that had not been extruded appeared to be somewhat leakier, but surface-adsorbed VCM by MLVs may account for the difference. The addition of FA-PEO-CH resulted in 20 percent increase in drug release, nevertheless the total amount of the drug remaining was > 60 percent even after a 2-h incubation period in SIF+BSC. Both DMPG- and DCP-containing liposomes behaved similarly after addition of FA-PEO-CH to the liposomes.

There are several reports that demonstrate the instability of liposomes in bile salt solutions, mentioned in Section 1.5.1. The main purpose of adding FA-PEO-CH conjugates to the liposomes was to improve GI affinity, since FA-PEO-CH had previously been identified as having potential for improving the transepithelial flux of liposomes across GI epithelia (Chap. 3). The results of this preliminary study have identified 200 nm DSPC:CH:DCP:FA-PEO-CH (3:1:0.25:0.05 m.r.) liposomes as a potential 'functionalized' oral delivery system for VCM with substantial drug loading (~40 percent) and demonstrating good stability under simulated GI conditions.

Integrity of the Caco-2 cell monolayer was established by immunofluorescent labeling of the ZO-1 tight junction protein (Figure 3-4) and by TER values of 562 ± 93 $\Omega\bullet cm^2$. Table 4-5 provides evidence of VCM and liposomal-VCM interaction with the cell monolayers. The transport of VCM across the monolayers was not quantifiable over the 2-h study period for all formulations tested, however, total recovery of VCM from basal and apical chambers indicated a substantial loss of VCM (~14 percent) after administration of FA-coated liposomes to the cells, suggesting that cellular uptake and intracellular trapping or metabolism of VCM had occurred. Previous work employing a large MW, hydrophilic, fluorescent Texas Red Dextran (3000 MW) as a marker in FA-coated liposomes gave rise to similar observations (Chap. 3), although in this case

Texas Red Dextran is a neutral molecule. It is as yet unclear whether VCM was metabolized or simply trapped intracellularly, reducing transepithelial flux of VCM. A better understanding of cellular processing and of enzymatic barriers to liposomes and liposome-associated molecules should facilitate improvements in the design of oral liposomes for therapeutic use. Until better in vitro models are designed for assessment of transepithelial colloid delivery, pre-clinical animal investigations are necessary for assessment of their therapeutic potential.

Table 4-1. Effect of pH and formulation technique on encapsulation efficiency of VCM in liposomes of various compositions.

| Liposome composition | EE[a] (%) pH 4.0[b] | | EE[a] (%) pH 7.0[b] | |
|---|---|---|---|---|
| | MLVs | DRVs | MLVs | DRVs |
| DSPC | n.r. | n.r. | n.r. | 58.82<br>(3.45) |
| DSPC:Chol (3:1 m.r.) | n.r. | n.r. | 10.35<br>(1.09) | 13.98<br>(2.33) |
| DSPC:Chol:DMPG (3:1:0.25 m.r.) | 6.21<br>(0.71) | 14.97<br>(4.56) | 7.78<br>(0.77) | 86.23<br>(3.99) |
| DSPC:Chol:DCP (3:1:0.25 m.r.) | 11.87<br>(0.78) | 86.05<br>(6.18) | 3.85<br>(0.58) | 96.74<br>(4.55) |

[a] Mean encapsulation efficiency (± s.d., $n \geq 3$)
[b] pH of drug-loading solution
n.r. = not reported

Table 4-2. Encapsulation efficiency of VCM in extruded DRVs (≤200nm) of various lipid compositions.

| Liposome composition[a] | EE[b] (%) |
|---|---|
| DSPC:Chol:DMPG (3:1:0.25 m.r.) | 5.52 (0.97) |
| DSPC:Chol:DMPG (3:1:0.5 m.r.) | 11.68 (2.73) |
| DSPC:Chol:DCP (3:1:0.05 m.r.) | 6.64 (1.10) |
| DSPC:Chol:DCP (3:1:0.25 m.r.) | 32.17 (4.38) |
| DSPC:Chol:DCP (3:1:0.5 m.r.) | 21.28 (1.37) |

[a] VCM added at drug:lipid 0.12 m.r.

[b] Mean encapsulation efficiency (± s.d., n=3)

Table 4-3. Effect of VCM concentration on encapsulation efficiency (%)[a] in negatively-charged DRVs (≤200nm).

| Initial VCM:lipid m.r. | DSPC:Chol:X[b] (3:1:0.25) | |
|---|---|---|
| | DMPG | DCP |
| 0.01 | 7.92 (1.73) | n.r. |
| 0.06 | 9.68 (3.89) | n.r. |
| 0.12 | 5.52 (0.97) | 32.17 (4.38) |
| 0.30 | n.r. | 26.59 (2.14) |

[a] Mean encapsulation efficiency (± s.d., n=3)
[b] X represents the negatively-charged lipid component.

Table 4-4. In vitro release studies of VCM liposome formulations in simulated GI fluids.

| Liposome composition[a] | % Remaining[b] | | | |
|---|---|---|---|---|
| | SGF | SIF | SIF+ | SIF+BSC |
| DSPC:Chol:DMPG[c] | 66.62<br>(10.45) | 68.14<br>(8.33) | 62.75<br>(7.22) | n.r. |
| DSPC:Chol:DMPG[d] | 70.98<br>(8.19) | 88.58<br>(16.05) | 87.64<br>(15.08) | 90.80<br>(5.48) |
| DSPC:Chol:DMPG:FPC[d,e] | n.r. | n.r. | n.r. | 65.73<br>(11.06) |
| DSPC:Chol:DCP[d] | n.r. | n.r. | n.r. | 82.48<br>(5.59) |
| DSPC:Chol:DCP:FPC[d,e] | n.r. | n.r. | n.r. | 66.80<br>(0.28) |

[a] 3:1:0.25 lipid m.r.

[b] Mean % liposome-associated VCM retained after 2 h at 37°C (± s.d., n≥3).

[c] unextruded liposomes

[d] extruded liposomes (<200nm diameter).

[e] FPC (FA-PEO-Chol) conjugate loaded at 0.05 m.r.

Table 4-5. Transport of liposomal VCM across Caco-2 monolayers.

| VCM Dosage Form | $P_{app}$ (cm/s) | % Loss Across Monolayer[a] |
|---|---|---|
| Solution, HBS pH 5.8 | n.q.[b] | 2.41 (1.07) |
| Uncoated liposomes | n.q. | -4.60 (3.62)[c] |
| FPC-coated liposomes | n.q. | 13.83 (0.94) |

[a] mean (± s.d., n=4)
[b] non quantifiable
[c] negative numbers denote concentration of VCM in apical chamber.

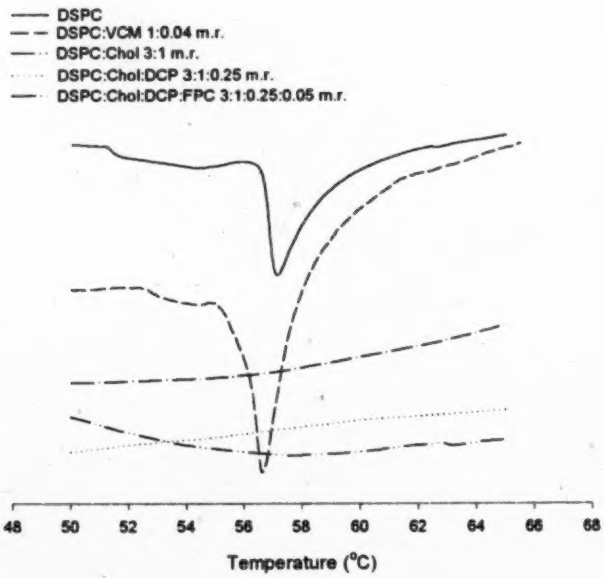

Figure 4-1. DSC thermograms depicting different lipid compositions showing the effect of VCM and Chol on the gel – liquid crystalline phase transition of DSPC liposomes.

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## *Chapter 5*

# IN VIVO EVALUATION OF A FOLIC ACID RECEPTOR TARGETED ORAL VANCOMYCIN LIPOSOMAL DOSAGE FORM

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## 5.1 Introduction

Based on the in vitro assessments described in Chapters 3 and 4, FA receptor-targeted liposomes have been identified as potential candidates for improved delivery of poorly-absorbed, water-soluble solutes. This study was proposed to determine the feasibility of oral delivery of VCM via FA receptor-targeted liposomes.

## 5.2 Background

Biotechnology and recombinant techniques have increased the available pool of potent proteins and peptides useful for a variety of indications. However, the availability of readily-usable oral dosage forms has not occurred at a comparable rate. Oral peptide vaccine development appears to be evolving rapidly due to the relative ease of delivery to the GALT, resulting in induction of mucosal immunity (1). Systemic delivery of proteins/peptides via the oral route, for example desmopressin or cyclosporin, has met with limited success due to the poor stability of proteins/peptides in the GI tract and to natural metabolic lumenal, brush-border, and cellular enzymatic barriers (2-4). In addition, specific physico-chemical properties of many proteins/peptides, such as hydrophilicity, charge, and large MW render them incapable per se of passive membrane absorption by enterocytes.

It appears that use of surface-labeled bioadhesive and receptor-mediated absorption promoting ligands can enhance systemic availability of molecules associated with colloidal dosage forms, including liposomes (5-8). In particular, the use of cellular nutrients as mediators of improved oral liposomal absorption is appealing due to the natural tendency of enterocytes to absorb molecules critical to the viability of body cells through energy-driven processes (9-11).

## 5.3 Materials and Methods

### *5.3.1 Materials*

See Section 4.3.1. Trifluoroacetic acid (TFA) was obtained from Sigma Chemical Co. (St. Louis, MO).

### *5.3.2 Liposome Preparation*

Liposomes were prepared according to Section 4.3.2.

### *5.3.3 Encapsulation Efficiency*

See Section 4.3.3.

### *5.3.4 In vivo protocol*

Male Sprague-Dawley rats (295.4 ± 13.2 g) were obtained from the Biosciences Animal Service (University of Alberta, Edmonton, AB, Can) and housed for at least 2 d in a clean room with access to food and water ad libitum. One day prior to an experiment, anesthesia of the rat was induced by pentobarbital (65 mg kg$^{-1}$) and a small incision was made over the right jugular vein. The vein was catheterized with Silastic® laboratory tubing (0.635 mm i.d., 1.194 mm o.d., Dow Corning Corp., Midland, MI, USA) containing heparinized (100 IU mL$^{-1}$) normal saline, and fixed in place with two nonabsorbable surgical sutures (Surgical Suture USP, Cyanamid, Montreal, QC, Can). Each cannula was terminated with a long piece of polyethylene tubing (PE-50, i.d. 0.58 mm, o.d. 0.965 mm, Clay Adams, Parsippany, NJ, USA) and the free end exteriorized to the dorsal side of the neck. The fasted rats were allowed to recover for 16 h following surgery and were allowed access to water ad libitum. Group I was administered an i.v. solution of VCM in Hepes buffered saline via the jugular vein cannula (6.85 mg $kg^{-1}$) and 0.25 mL blood samples were drawn at times –0.5, 2, 5, 15, 30, 45, 60, 90, 120, and 180 min post-administration. Groups II, III, and IV were orally-administered a solution of VCM in Hepes buffered saline (61.75 mg $kg^{-1}$), a suspension of uncoated liposome-entrapped VCM (62.4 mg $kg^{-1}$), or a suspension of FPC-coated liposome-entrapped VCM (47.69 mg $kg^{-1}$) via an oral gavage tube. Subsequently, 0.25 mL blood samples were drawn at –0.5, 15, 30, 60, 90, 120, 180, 240, 300, and 360 min post-administration. Blood samples were immediately centrifuged at 5000 rpm for 5 min, and the plasma was collected and stored at -20°C. Long-term VCM stability (> 1 y) in plasma at -20°C has previously been demonstrated (12). The total blood volume drawn from each rat represented approximately 12.5 % of the blood volume available. The total fluid volume administered was 0.2 mL for i.v. dosing and 2.0 mL for oral dosing. The total lipid load in oral dosing of liposomes was approximately 0.30 mmol. The Guide to the Care and Use of Experimental Animals of the Canadian Counsel on Animal Care was followed throughout.

### *5.3.5 Chromatographic Analysis*

High-performance liquid chromatography (HPLC) coupled with UV detection (Gilson 117, Middleton, WI, USA) at 250 nm was used to quantitate VCM. Mobile phase of Sørenson's phosphate buffer (pH 7.0):acetonitrile (9:1, v/v) was degassed and pumped through a 5 μm reverse-phase C-8 column (125 x 4mm LiChrospher 60 RP-Select B, Merck, Germany) at a flow-rate of 1mL/min. All reagents were HPLC grade.

Prior to analysis, plasma samples (100 μL) were treated with 300 μL 10% TFA:methanol (2:1, v/v), vortexed, and centrifuged at 14000 rpm for 10 min (Eppendorf model 5415 microcentrifuge, Germany) to remove plasma proteins. The supernatant was collected and fresh buffer solution was added to a volume of 500 μL (4-fold sample dilution). Samples (100 μL) were injected by an auto-sampler (Basic Marathon, Spark Holland, Netherlands). Peak areas of VCM were quantitated using the Data Ally™ (Lab Alliance, PA, USA) data acquisition program. VCM concentrations were calculated from a standard calibration curve (2-50 μg $mL^{-1}$) prepared daily. The limit of VCM detection in plasma was 2 μg $mL^{-1}$, in agreement with other reports (12-14).

### *5.3.6 Pharmacokinetic (PK) Analysis*

Standard PK parameters were obtained from individual rat plasma concentration – time profiles of VCM calculated according to the standard non-compartmental analysis in WinNonlin Standard Edition version 1.0 (SCI, Apex, NC, USA). Area under the plasma concentration – time curve (AUC) was calculated using the linear trapezoidal rule. Absolute bioavailability (F) was calculated according to:

$$F = \frac{AUC_{test}^{0-last}}{AUC_{iv}^{0-last}} \times \frac{Dose_{iv}}{Dose_{test}} \qquad (2)$$

where $AUC_{test}$ and $Dose_{test}$ represent the respective mean oral dosing parameters of groups II, III, and IV.

### *5.3.7 Statistical Analysis*

PK parameters were assumed to follow a log-normal distribution (except for $t_{max}$), and were log-transformed prior to statistical analysis. AUC and $C_{max}$ were dose-normalized prior to statistical analysis. All parameters were recorded as arithmetic means (± s.d.).The PK parameters obtained were analyzed by independent measures one-way analysis of variance (ANOVA), and a *post hoc* Student-Newman-Keuls (SNK) test was used to determine where, if any, differences occurred. A value of $p \leq 0.05$ was considered statistically significant. The computer program SigmaStat 2.0 (SPSS, Chicago, IL, USA) was used for all statistical procedures.

## 5.4 Results

The EE of VCM in DSPC:CH:DCP (3:1:0.25 m.r.) DRVs was 32.0 ± 4.4 percent and was reduced to 25.0 ± 6.4 percent following liposome coating. The average particle size of the liposomes was 208.75 ± 8.85 nm and 291.67 ± 20.01 nm for uncoated and FPC-coated liposomes, respectively.

The PK parameters generated from non-compartmental analysis are reported in Table 5-1. Groups not connected by lines are statistically significant from each other. The i.v. parameters correlated with those of previous PK reports for VCM dosing in rats (13;15); VCM exhibiting two-compartmental kinetics with a rapid distribution phase and a terminal half-life of approximately 50 min. The total body clearance of VCM was 1.13 mL $min^{-1}kg^{-1}$.

Figure 5-1 illustrates the mean plasma concentration – time profiles for all dosage forms tested (i.v. data inset). AUC from time 0-last were reported and utilized for bioavailability. Dose-normalized AUC was significantly different for all dosage forms tested ($p < 0.05$). Based on the mean data, the absolute bioavailability of VCM was 1.74 percent, correlating well with previous reports (13). The bioavailability of uncoated liposomal VCM was 6.7 percent representing a mean 3.9-fold relative increase compared to the VCM solution. The bioavailability of FPC-coated liposomal VCM was 21.8 percent representing a mean 12.5-fold increase compared to the VCM solution. Folic acid adsorbed at liposome surfaces enhanced the performance of the liposomal delivery system by 320 percent. The $C_{max}$ was significantly different for all dosage forms tested whereas the $t_{max}$ showed no statistical differences. There appeared to be a multiple-peaking phenomenon occurring after oral liposome dosing, which made calculation of AUC $0-\infty$ difficult.

## 5.5 Discussion

The suggestion that liposomes may serve as oral delivery vehicles for poorly-absorbed water-soluble agents such as proteins or peptides remains controversial even though evidence of protection of encapsulated agents from the harsh environment of the GI tract is convincing (11;16). Bioavailability of liposome-encapsulated agents is generally dependent on a series of inter-related factors, including liposome loading, dose, amount retained at absorption site(s), anatomy and physiology of the absorption site(s), effect of surface-modification, and the PK involved

Attention to liposome stability in the milieu of the GI tract, including the use of high $T_m$ lipids, and the use of novel targeting ligands, such as FA, to mediate the uptake of these dosage forms have previously demonstrated potential for improving the transport of poorly-absorbable, water-soluble agents across Caco-2 monolayers (Chapter 2,3). In addition, control of particle size appears to be a relevant factor for oral microparticulate delivery, optimal sizes of 50-500 nm being a pre-requisite for successful delivery (6). In particular, VCM is an excellent model for assessing the in vivo absorption-promoting effects of liposomes since it is representative of the peptide class of compounds (poorly-absorbed), is stable within the GI tract, and does not undergo hepatic biotransformation to any significant extent.

It is, as yet, undetermined whether the multiple-peaking phenomenon prevalent for liposome systems is a statistically significant phenomenon due to the high variability in plasma concentrations. However, on inspection of individual rat profiles (c.f. Appendix I) the multiple-peaking phenomenon consistently appears for liposome-dosed rats. Multiple-peaking due to pH-dependent solubility, biotransformation, or enterohepatic recirculation of VCM can be ruled out, based on previous VCM PK reports (17). Enterohepatic recirculation of liposomes (colloids) or lymphatic absorption, e.g. via Peyer's Patches, may have implications for the occurrence of multiple VCM peaks (18-20). However, the larger magnitude of ensuing peaks is suggestive of a pre-absorptive phenomenon possibly related to delayed gastric emptying due to lipid dosing or complexation (precipitation) of VCM with DCP at low pH. Regardless, FPC-coated liposomes significantly improved (3.2-fold) the delivery of VCM compared to uncoated liposomes at equivalent lipid loads.

Surface-modification of microparticles with targeting ligands (lectins) has previously been employed to demonstrate the potential of improving colloidal-particulate absorption by 'shifting' absorption sites from lymphatic to non-lymphatic GI tissue (8). Other reports have alluded to the trapping of liposomes at pre-systemic sites after oral delivery, providing sustained release depots of entrapped protein (21). Presently, utilization of vitamin-targeting strategies improved the absorption of liposome-associated VCM via folate receptor-mediated endocytosis, suggesting that this delivery system could have functional possibilities for oral protein/peptide delivery, similar to reports of successful cyanocobalamin-mediated uptake of microparticulates (22). Further developments will require confirmation with other like agents and a larger sample size.

Table 5-1. PK parameters of VCM following i.v. and oral dosing.

| PK parameters | i.v.[a] (I) | Soln[a] (II) | Uncoated Liposomes[a] (III) | Coated Liposomes[a] (IV) | Statistical Comparison[b] |
|---|---|---|---|---|---|
| $AUC_{0\text{-last}}/D$ ($mL^{-1}$ min $kg^{-1}$) ($\times 10^{1}$) | 6.42<br>(1.05) | 0.11<br>(0.04) | 0.43<br>(0.27) | 1.40<br>(0.86) | I II III IV |
| $C_{max}$ ($\mu g\ mL^{-1}$) | 325.11<br>(38.00) | 11.35<br>(2.61) | 25.14<br>(13.06) | 42.21<br>(21.96) | I II III IV |
| $t_{max}$ | 0 | 80<br>(17.32) | 210<br>(114.89) | 112.5<br>(61.85) | $\underline{\text{II III IV}}$ |

[a] mean (s.d., n=4)

[b] All numbers were log-transformed prior to statistical analysis. Formulation numbers joined by a line are not significantly different (one-way ANOVA, SNK, $p < 0.05$).

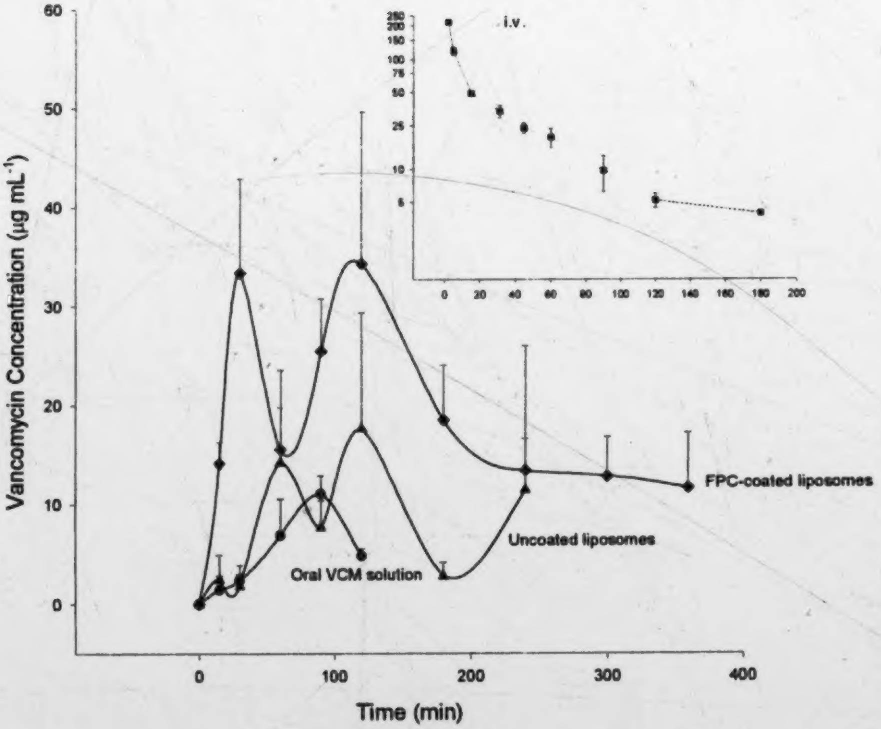

Figure 5-1. In vivo disposition of VCM after i.v. administration (inset), ■, and oral administration of FPC-coated liposome-entrapped VCM, ◆, uncoated liposome-entrapped VCM, ▲, and VCM solution, ●. Error bars represent s.d., n=4.

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## *Chapter* 6

# GENERAL DISCUSSION AND CONCLUSIONS

## 6.1 Historical

The first encouraging evidence regarding improved insulin delivery following oral liposome delivery advocated by Gregoriadis et al. and Ryman et al. circa 1976 (1;2) has not resulted in the development of a marketable entity in the past 20 y. Numerous contradictory reports have resulted in much skepticism towards the merit of liposomes as useful oral dosage forms. By the mid 1980's Chiang and Weiner (3;4) left little hope for the development of oral liposomes following extensive mechanistic and in vivo investigation. In retrospect, many of the findings, both positive and negative, were not predicated on specific drug delivery objectives nor were they adequately explained by the mechanistic information available. In accordance, recent oral liposome designs have optimized lipid content, particle size, and surface coatings for improving stability and bioadhesion in the GI tract, resulting in positive reports of gastrointestinal uptake (5-11). An improved understanding of liposome (and associated drug) interactions with the intestine at cellular levels should enable improvements in the utility of oral liposomes in the future.

## *6.2 General Discussion*

Considerations of stability of liposomes in the GI tract have resulted in a number of proposed designs for enhancing liposome integrity including the use of inherently stable phospholipids (high $T_m$), polymer-coating, polymerizable lipids, and microencapsulation (12). In order to simplify the formulation process in producing stabilized systems, a high $T_m$ phospholipid platform was chosen for developing oral liposomes. The choice of liposome membrane constituents depends on a good understanding of the physical behavior of lipid compositions and the effects of polymeric additives in the bilayers. A simple imaging technique was described in Chap. 2 for determining the interfacial behavior of phospholipid monolayers at an oil-water interface in the presence of various additives. The oil-water interface is considered a more relevant interfacial model than surface-films for bilayer investigations (13). Surface pressure ($\pi$) measurements at the chlorobenzene-water interface yielded clues of the types of lipid interactions that could also be determined from calorimetry experiments in bilayer-water systems (14). Thus, surface pressure indicies were used to quantify the extent of interfacial condensation or expansion following polymer or lipid adsorption at the interface, and polymer impact ratios ($P^*$) gave a measure of the overall effects of a polymer on the monolayer. For instance, the $P^*$ of CM-chitin indicated weak interactions with the bilayer whereas CHM interacted more intensely as a result of penetration of its 'hydrophobic anchor' (terminal CH on an AECM spacer) in the bilayer, a condition that has been suggested as a pre-requisite for coating liposomes (15-18). Although this approach of physicochemical characterization of a bilayer has not been attempted previously, it offers a fairly simple means of evaluating the impact of additives on the integrity of bilayers, which are essentially two molecular monolayers.

Presentation of FA to absorptive enterocytes was hypothesized to improve the GI uptake of liposomes and contents, a strategy exploiting natural receptor-mediated endocytotic pathways. In order to accomplish this a folic acid construct of FA-PEO-CH was prepared and adsorbed on liposomes (Chap. 3). However, when this occurred the surface coating reduced the EE of VCM in liposomes by 20 percent and, furthermore caused a 2-fold increase in VCM release rate in the presence of a bile-salts solution compared to uncoated liposomes. PEO-cholesterol ethers have previously been shown to induce a concentration-dependent leakage of entrapped-calcein from SUV liposomes (19). Conversely, investigations of the effect of cholesterol-derivatized polysaccharide coatings of liposome surfaces resulted in improved liposome stability on exposure to plasma or simulated intestinal fluids (15;20-24). These variable effects of different polymer-coatings of liposomes are possibly related to the surface activity of the 'hydrophobized' polymeric derivative, molecular weight of the derivative, and the added concentration, resulting in either homogenous or phase-separated lamellar phases or mixed miceller phases (25). However, DSC thermograms of FA-PEO-CH-coated liposomes indicated no phase separation at 1.2 mole percent incorporation. Enhanced liposome leakage may possibly be attributed to formation of open bilayer discs, as described similarly for PEG-PE incorporation at sub-micellar concentrations (26). Notwithstanding, there is other evidence that CH incorporation at 30 mole percent of total lipids counteracts mixed micelle formation under the influence of low molecular weight PEG-PE (27), a finding consistent with the optimized targeted-liposome formulation in these works containing 25 mole percent CH (c.f. Chap. 4). The effect of varying amounts of FA-PEO-CH added to liposomes, although not specifically investigated, is expected to result in only minor alterations in liposome behavior. Based on lipid:PEG ratios (mol/mol), it has been shown that 2 mole percent of 1 kD and 1 mole percent of 2 kD DSPE-PEG, was enough to completely coat (i.e. protect) liposome surfaces (28), and concentrations above 7 mole percent of 2 kD PE-PEG can induce mixed micelle formation (27). Although FA-PEO-CH slightly reduced liposome stability in simulated biological fluids, exposure of FA-targeted liposomes to intestinal Caco-2 cell culture significantly enhanced the uptake of TR-dex and VCM presumably by a specific FA receptor-mediated mechanism. Previous reports have argued that receptor-mediated cellular uptake appears to be unaffected by inclusion of the PEO-CH adjunct. Conversely, fluid-phase endocytosis can be significantly inhibited (29;30). Unfortunately, limitations of current cell culture models have prevented a clear understanding of the manner by which liposomes modulate transcytosis in vivo, since colloidal-particle absorption and receptor-mediated processes are often not well-represented in vitro. Regardless, the cell-culture investigations enabled functional evaluation of FA-mediated drug delivery at a cellular (intestinal tissue) level, indicating the potential of exploiting natural vitamin receptor-targeted delivery systems.

The question of what method of liposome preparation would be both convenient and yield liposomes of the desired characteristics was addressed (Chap. 4). Ultimately, the DRV technique offered the best conditions for the greatest level of encapsulation of VCM, consistent with reports advocating this technique for improving the EE of water-soluble solutes (31). The DRV method avoids exposure of the peptide/protein to organic solvent, in contrast to the REV method even though comparable EEs are possible (32). Freeze-and-thaw MLVs have been shown to improve liposome loading of Ara-C (23), a water-soluble solute, however the freezing process may be detrimental to proteins in particular. Recently, the DRV process was reported to be successful in improving the EE of active insulin in liposomes (18), again illustrating the advantages of DRVs for labile materials.

Particle size control was considered to be an important factor in liposome design, underscored by published evidence of in vivo uptake of colloidal particles only between 50-500 nm in diameter (33), a condition often overlooked in previous investigations. The designed liposome formulation, DSPC:CH:DCP:FA-PEO-CH (3:1:0.25:0.05 m.r.) (Chap. 4), prepared in the 200 nm size range by extrusion filtration was found to have as much as 32 and 25 percent EE of VCM for non-targeted and targeted liposomes, respectively. As well, the liposomes were reasonably stable in simulated intestinal fluid containing 10 mM mixed-bile salts. Previous reports have attested to the instability of liposomes to bile salt solutions (34-39), but these have generally not emphasized liposomes of high $T_m$ phospholipids which are generally more stable (40;41) making them good candidates for oral liposome delivery.

Mechanistic information derived from cell culture data have suggested that liposomes are processed intracellularly causing entrapped drug to be released. In situ intestinal loop experiments have supported this conclusion (42). At first glance there appeared to be a discrepancy between liposome-entrapped TR-dex versus VCM transport. However, after closer inspection it appeared that the different behaviors of the respective agents is likely due to differences in cellular processing of the individual compounds rather than differences in cellular handling of liposome delivery systems. Experimentally, the filters supporting Caco-2 cell growth were themselves formidable barriers to liposome transport, preventing appearance of liposomal-TR-dex in the basolateral compartment. Such a phenomenon has also been reported for lectin-mediated nanoparticle transport across Caco-2 cell culture (43). However, when a confluent cell monolayer was present, liposomal-TR-dex transport occurred (Chap. 3). If this same phenomenon had prevailed with liposomal-VCM transport, then VCM should have been detected in the basal chamber, but this did not occur. Hence, it was deduced that VCM either remained trapped or it was metabolized in the cells. If this is the case, liposomes must also be trapped or digested intracellularly. Extensive investigations of folate-mediated cellular uptake in other cell lines have indicated that targeted liposomes become encased in non-lysosomal endosomal compartments at an average pH of ~5 (44). At this pH VCM would be positively-charged, which may generate insoluble complexes of VCM with the negatively-charged lipid components (e.g. DCP) although this was not determined. The effects of endosomal disrupting agents on transcytotic delivery of liposome-entrapped drugs should be further investigated (45;46). More detailed studies should elucidate relationships between FA-mediated uptake and cellular routing, perhaps identifying novel approaches towards quantitative transcellular delivery. Moreover, on a macroscopic level, the relationships between GI pH and enterocyte FA receptor expression on FA-mediated drug delivery needs to be clearly delineated. Nevertheless, these studies have demonstrated targeting due to liposomal surface FA and, presumably subsequent internalization of the encapsulated agent in cells. Factors affecting transport were clearly different for TR-dex and VCM. Whether baso-lateral transport of entrapped compounds is mediated by liposome association remains unclear. Although current cell culture techniques allowed for some mechanistic interpretation of drug delivery at the cellular level, precautions must be taken in extrapolating to in vivo because of the: i) relatively high TER, ii) differential expression of cell proteins (enzymes, receptors), iii) lack of tissue differentiation, including multiple cell types (e.g. M-cells, lymphatic tissue), iv) absence of basal lymphatic or systemic circulature, and v) presence of a non-physiologic barrier to the transport of colloids. As a preliminary trial, the Caco-2 cell culture enabled a perspective look at the cell interaction differences among the formulations. It was shown that FA-PEO-CH-coated liposomes altered the transport behavior of VCM in possibly a favorable manner. New developments in cell culture methodology such as mucus-secreting intestinal co-cultures (47) and cell lines which express lymphoid tissue (48) should prove useful in future investigations of such delivery systems.

Administration of liposomal-VCM to the rat improved bioavailability ~4-fold, compared to an oral VCM solution. The targeted liposomes increased VCM delivery by 320 percent compared to conventional liposomes and 12.5-fold compared to the VCM solution. Recent works have similarly described the oral delivery of erythropoietin and insulin in 100 nm 'rigid' vesicles finding ~1–30 percent bioavailability, as well as confirming size-dependent uptake (7;8). Notably, it was reported that prolongation of these agents in plasma was due to their association with vesicles. This could occur if liposomes were either trapped at a pre-systemic site or processed via lymphatic routing. As identified In Chap. 5, the difficulty in measuring the VCM $\lambda_{el}$ and the occurrence of VCM multiple plasma peaks suggests that liposomes (targeted and non-targeted) can demonstrably change VCM kinetics and, perhaps, give proteins/peptides access to previously inaccessible absorption routes, e.g. lymphatic or transcellular routing. In this regard, VCM (drug) absorption is not completely determined by physical parameters (e.g. dissolution rate) but also by physiologic determinants involving absorptive pathways. Liposome-surface characteristics play a significant role in the nature of this routing and, consequently, exploitation of FA - receptor-mediated endocytosis should be encouraged as a route for oral liposome drug delivery. Other works have also confirmed that carbohydrate surface coating results in the successful oral delivery of liposome-encapsulated insulin (11;49). It is expected that continued identification of physiologic controls that can be exploited or modulated by formulation components will result in less variable delivery profiles, a pre-requisite for successful commercial development of this type of drug delivery system. Specific formulation approaches and targeted delivery objectives in conjunction with a sound understanding of the physiology of the GI tract are needed and, in this regard, a targeted oral liposome system possesses merit for further development.

### *6.3 Conclusions*

1. ADSA is a useful imaging technique for elucidating the interactions of lipid and polymeric additives in a monolayer. Surface pressure indicies

($\pi^*$) allowed quantitation of the effect of additives, and polymer impact ratios ($P^*$) expressed the overall effects of polymer interaction with the monolayer, whereby the preferred properties of a polymer for coating liposomes could be ascertained.

2. The DRV technique is superior to traditional hydration methods with respect to the EE of water-soluble solutes in liposomes, e.g. VCM. A 25-fold improvement in EE by DRVs was found compared to MLVs.
3. Liposomes composed of high $T_m$ lipids offered good stability in simulated intestinal fluid containing 10 mM bile salts. Eighty-two and 67 percent of VCM remained entrapped after 2 h of exposure at 37°C.
4. Folic acid - PEO derivatives effectively modified the cellular uptake of liposome-associated compounds. Targeted liposomal-TR-dex flux increased 5-fold and VCM uptake increased 5.7-fold in Caco-2 cell culture compared to their respective solutions.
5. Conventional cell culture studies provide good mechanistic information of uptake and transport in a cell layer, but caution is necessary in attempting to extrapolate transport results to the in vivo condition.
6. Oral liposome systems have potential for improving the bioavailability of proteins / peptides or other poorly-absorbed, water-soluble solutes based on the VCM data. Non-targeted liposomes improved the bioavailability of VCM 3.9 fold compared to an oral solution, resulting in 6.7 percent of the dose being absorbed (c.f. 1.7 percent).

7. FA-targeted liposomes have potential for further enhancing the bioavailability of proteins/peptides or other poorly-absorbed, water-soluble solutes based on the VCM data. FA-targeted liposomes improved the bioavailability of VCM 12.5-fold compared to an oral solution, resulting in 21.8 percent of the dose being absorbed (c.f. 1.7 percent).

### *6.4 Future Perspectives*

A pragmatic perspective of oral liposome literature shows that considerable progress has been made in the last 12 years towards the development of liposomes as useful oral dosage forms, even though there are currently no marketable entities. The challenges of liposome formulation have consistently been outweighed by the potential therapeutic benefits for oral delivery of inherently unstable drugs (e.g. proteins), poorly-soluble drugs (e.g. griseofulvin), vaccines, and drugs which have untoward local toxicities (e.g. NSAIDs).

A paradigm shift in the realization of nano- and microparticulate uptake by M-cells (50) has resulted in a resurgence in oral liposome technology (5-11;51-58). A broad interpretation of the data leads to the following conclusions: i) particulate uptake via M-cells does occur and appears to be a viable route for vaccine delivery, ii) sub-micron particle sizes in the 50 - 500 nm range produce the optimum response, iii) negatively-charged liposomes have an affinity for M-cells, iv) surface modification of particulates can attenuate M-cell regioselective delivery, v) interspecies variabilities of uptake capacity and M-cell prevalence in animals limits extrapolation of results to human. Hence, drug delivery to PPs will require tighter control of targeting and transport and, at least for now, will likely be reserved for vaccine delivery.

The design of reproducible oral liposome systems for systemic therapies currently presents the greatest challenge to liposome formulators. Problems encountered in attempts to deliver therapeutic quantities of liposomal drug to lymphatic tissue are: i) a variable extent and rate of absorption and, ii) the possibility of inducing an immune response against the delivered agent (a desirable outcome for vaccines). It appears that a strategy of using selective targeting moieties (e.g. lectins) can shift colloidal absorption to non-lymphatic tissue sites, allowing greater access of the formulation and contents to the mesenteric vasculature, leading to systemic absorption (59;60). Exploitation of endogenous receptors such as cyanocobalamin receptor-mediated uptake of nanoparticles has been successfully achieved (61). Notwithstanding the inherent differences between liposomes and nanoparticulates, this thesis has described a similar improvement in systemic absorption of a liposome-entrapped drug by exploiting FA receptor-mediated delivery. Future investigations should attempt to improve specificity of targeting for either lymphatic or systemic disposition (50), depending on the course of treatment (62). Cell-culture, particularly the Caco-2 cell line, may be a good mechanistic model in the early evaluation of liposomes as well as other microparticles (63). Cell culture methodology is improving with the advent of non-transformed cell-lines and specific models of intestinal cell subtypes (e.g. M-cells) (48) which enable in vitro evaluation of interactions between liposomes and cells in the GI tract. The evolution of more porous, hydrophilic matrices for cellular adhesion may improve the current cell culture hardware allowing better evaluation of colloidal absorption.

Historically, liposomes lacked stability within the GI tract, however with new coating technologies, polymerizable lipids, and inherently more stable liposomes, such as archaeosomes derived from archael bacteria (64), the problem of stability in the GI tract can be minimized. Progress in oral liposome technology now depends on improving affinity to the GI tract in a reproducible manner. Perhaps polymers used to enhance liposome stability can also provide targeting to intestinal sites. Polymers that become firmly attached to the liposome surface without compromising liposome integrity are required. Models that are able to elucidate the exact mechanisms of polymer functionality at the cell level need to be identified, particularly to unravel the discrepancies reported for PEGs at liposome surfaces (65;66). Interestingly, oral liposome delivery development may be advanced from an understanding of the functionality of bacterial membranes in the crossing of epithelial surfaces. An understanding of the ability of glycoproteins (e.g. lectins, toxins) to transport across epithelia would improve chances for success in oral liposome delivery (67). Agents also need to be identified that can be used to induce endocytosis after binding (68). Alternatively, newly identified tight-junctional integral membrane proteins, such as junctional adhesion molecule (JAM protein), which have been identified as modulating leukocyte migration through paracellular pathways could be exploited for drug delivery technologies (69). Interestingly, these data suggest that the paracellular route is not limited to small molecules and can be regulated (70), in contrast to conventional thinking (71;72). A rekindled interest in intrinsic cell-membrane binding proteins specific for cyanocobalamin, folic acid, and bile salts have stimulated new ideas for oral drug delivery. Liposome surfaces can be constructed to accommodate an assembly of binding vectors acting concertedly to produce an endocytotic event, particularly for ligands with binding proteins associated with caveolae. Utilization of targeting strategies and the liposome depot effect would appear to have therapeutically relevant possibilities for oral liposome administration. Simplicity of design will be a key factor for the development of commercial entities. As it stands the oral liposome story is in its infancy.

## 6.5 References

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# Appendix I.

## Raw data: in vivo investigation

i.v. VCM

| Time | Rat 1 | Rat 2 | Rat 3 | Rat 4 | mean | s.d. |
|---|---|---|---|---|---|---|
| 2 | 196.18 | 214.79 | 239.16 | * | 216.71 | 21.56 |
| 5 | 114.29 | 105.19 | 136.05 | 116.72 | 118.06 | 12.98 |
| 15 | 51.67 | * | 45.82 | 53.65 | 50.38 | 4.07 |
| 30 | 35.81 | 39.50 | 25.75 | 31.46 | 33.13 | 5.92 |
| 45 | 21.52 | 28.19 | 21.10 | 22.55 | 23.34 | 3.29 |
| 60 | 14.50 | 26.77 | 17.04 | * | 19.44 | 6.48 |
| 90 | 13.86 | * | * | 12.85 | 13.35 | 0.72 |
| 120 | 6.12 | 15.95 | 15.95 | * | 12.67 | 5.67 |
| 180 | 3.67 | 14.83 | 14.83 | 11.00 | 11.08 | 5.26 |
| Dose | 6850 ug kg-1 | | | | | |

Oral VCM solution

| Time | Rat 1 | Rat 2 | Rat 3 | Rat 4 | mean | s.d. |
|---|---|---|---|---|---|---|
| 15 | 4.28 | 0.00 | 0.00 | 1.82 | 1.53 | 2.03 |
| 30 | 2.13 | 5.13 | 0.00 | * | 2.42 | 2.58 |
| 60 | 12.28 | 8.35 | 0.00 | 6.68 | 6.83 | 5.12 |
| 90 | 12.57 | 7.28 | 13.12 | * | 10.99 | 3.23 |
| 120 | 5.96 | 4.93 | 3.45 | 2.46 | 4.20 | 1.55 |
| 180 | 0.00 | 0.00 | 0.00 | 0.00 | 0.00 | 0.00 |
| 240 | 0.00 | 0.00 | 0.00 | 0.00 | 0.00 | 0.00 |
| 300 | 0.00 | 0.00 | 0.00 | 0.00 | 0.00 | 0.00 |
| 360 | 0.00 | 0.00 | 0.00 | 0.00 | 0.00 | 0.00 |
| Dose | 61735 ug kg-1 | | | | | |

* sample not recorded

Uncoated Liposomal VCM

| Time | Rat 1 | Rat 2 | Rat 3 | Rat 4 | mean | s.d. |
|---|---|---|---|---|---|---|
| 15 | 0.00 | 9.79 | 0.00 | 0.00 | 1.96 | 2.45 |
| 30 | 0.00 | 2.43 | 4.81 | 0.00 | 1.45 | 1.81 |
| 60 | 13.25 | 16.48 | 0.00 | 27.10 | 11.37 | 14.21 |
| 120 | 2.34 | 3.74 | 12.10 | 12.46 | 5.11 | 7.66 |
| 180 | * | 0.00 | 8.56 | 44.25 | 13.20 | 17.60 |
| 240 | * | 0.00 | 3.31 | 5.21 | 9.12 | 2.84 |
| 300 | 16.98 | 0.00 | 22.85 | 5.75 | 2.13 | 11.40 |
| 360 | 0.00 | 0.00 | * | 0.00 | 0.00 | 0.00 |
| Dose | 62412 ug kg-1 | | | | | |

FA-coated liposomal VCM

| Time | Rat 1 | Rat 2 | Rat 3 | Rat 4 | Mean | s.d. |
|---|---|---|---|---|---|---|
| 15 | 16.14 | 16.90 | 7.59 | 15.74 | 16.32 | 0.82 |
| 30 | 13.43 | 40.54 | 23.09 | 56.25 | 48.40 | 11.10 |
| 60 | 3.39 | 0.00 | 27.79 | 30.92 | 15.46 | 21.87 |
| 90 | * | 37.27 | 17.04 | 22.12 | 29.69 | 10.71 |
| 120 | 25.06 | 38.13 | 0.00 | 73.68 | 55.91 | 25.13 |
| 180 | 3.12 | 19.70 | 29.57 | 21.48 | 20.59 | 1.26 |
| 240 | 2.48 | 0.00 | 0.00 | 50.99 | 25.50 | 36.06 |
| 300 | 1.02 | 18.34 | 15.87 | 15.80 | 17.07 | 1.80 |
| 360 | 0.00 | 25.75 | 6.18 | 14.46 | 20.10 | 7.98 |
| Dose | 47689 ug kg-1 | | | | | |

*sample not recorded.

The individual chapters of this thesis have also been published in international pharmaceutical journals, or have been submitted.

## *Research articles*

Anderson K.E., Eliot L.A., Rogers J.A. In vivo Evaluation of Folic Acid Receptor Targeted Oral Vancomycin Liposomal Dosage Forms. Pharmaceutical Research (submitted, 1998)

Anderson K.E., Stevenson B.R., Rogers J.A. Formulation and In Vitro Evaluation of Targeted Oral Vancomycin Liposomal Dosage Forms. Pharmaceutical Research (submitted, 1998).

Anderson K.E., Stevenson, B.R., Rogers J.A. Folic Acid-PEO-Labeled Liposomes to Improve GI Absorption of Encapsulated Agents. Journal of Controlled Release. (Submitted, 1998).

Rogers J.A, Anderson K.E. The Potential of Liposomes in Oral Drug Delivery. Critical Reviews in Therapeutic Drug Carrier Systems. (accepted for publication, 1998).

Anderson K.E., Rogers J.A., Li D. An Interfacial Tension Model of the Interaction of Water-Soluble Polymers with Phospholipid Composite Monolayers. Journal of Pharmacy & Pharmacology (1997) 49: 587-591.

## *Abstracts*

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Anderson K.E., Stevenson B.R., Rogers, J.A. Improved transport of folic-acid linked PEO-conjugates across Caco-2 monolayers. Proc. Int. Symp. Control. Rel. Bioactive Mat. 25th ed. Las Vegas, NV, 1998, p 922-3.

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Anderson, K., Rogers, J.A., and Li D. *Measurement of the Surface Tension of Liposomes.* American Association of Pharmaceutical Scientists Annual Convention, Miami, FL, November 1995. Pharmaceutical Research Supp. 12(10).